STUDIES ON THE URINARY α-KETOLIC CORTICOSTEROIDS

by

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GENERAL INTRODUCTION

Among the urinary steroids those possessing an α-ketolic side-chain (CH_2OH-CO-) attached to C-17 in ring D of the nucleus are considered to have special significance. All seven active compounds so far isolated from the adrenal cortex present this feature together with a ketonic group at C-3 and[a] double bond between C-4 and C-5. It is generally held that the quantitative and qualitative analysis of the urinary α-ketolic steroids can be a valuable if indirect tool to assess adrenocortical function, because these metabolites bear a more direct connection with the hormonal precursors than those steroids in which the side-chain has been removed or altered in the course of the metabolism.

A list of the α-ketolic steroids isolated from the adrenal gland can be found in the book by Dorfman and Ungar (1953). A new item in it is aldosterone. The story of its isolation and identification is given with many references in a review by Wettstein and Anner (1954). To the urinary/

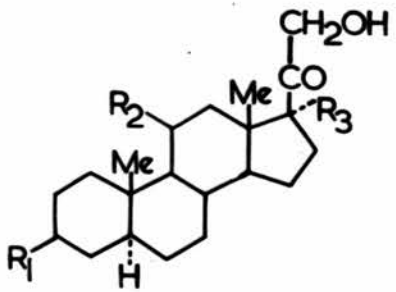
urinary α-ketolic steroids included in the above-mentioned book it is necessary to add pregnane-3α,17α,21-triol-20-one, isolated by Rosselet, Overland, Jailer and Lieberman (1954) from the urine of two women suffering from Cushing's syndrome due to adrenal carcinoma. This finding has been confirmed by Touchstone, Richardson, Bulaschenko, Landolt and Dohan (1954). Romanoff, Wolf and Pincus (1952) reported the presence in urine of allopregnane derivatives with no further confirmation. Their inclusion in Table 1 is merely provisional. There have been recent reports (Richardson, Touchstone and Dohan, 1954, 1955) of the identification in urine, after the administration of the six active adrenal hormones (aldosterone excepted), of the corresponding tetrahydro derivatives, i.e., with the α,β-unsaturated ketonic group in ring A fully reduced. Moreover, the same group of workers has identified the tetrahydro derivatives of corticosterone and 11-dehydrocorticosterone, allotetrahydrocorticosterone, and corticosterone in human urine after parenteral administration of ACTH (Touchstone, Bulaschenko, Richardson and Dohan, 1954).

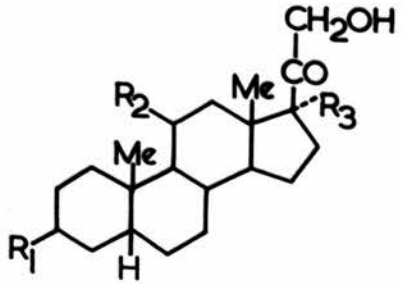
All these α-ketolic steroids are listed in Table /

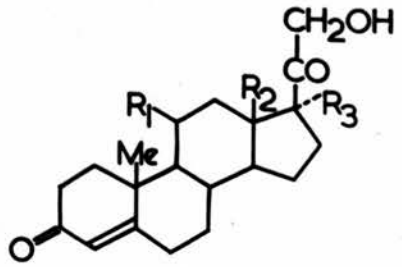
Table 1 and their formulae given in Fig. 1.

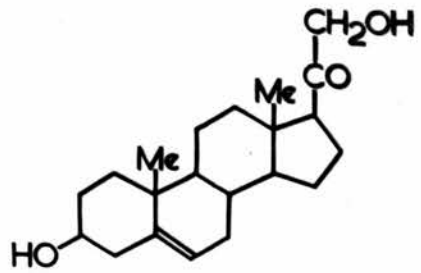
Burstein, Dorfman and Nadel (1954) have described the isolation of 6β-hydroxycortisol from the urine of a man with Cushing's syndrome who was given 275 mg. of cortisol intravenously after removal of the diseased adrenal. The compound was also detected in late human pregnancy urine. This steroid has not been included in the Table or in Fig. 1 because the significance of the 6β-hydroxylation in normal persons is still not clear.

It can be seen that only four of the seven active cortical compounds have been found in urine: hydrocortisone, cortisone, aldosterone and corticosterone. A common metabolic process seems to be the reduction of ring A to give the saturated pregnane derivatives. It is remarkable that only allopregnane and no pregnane derivatives have been isolated from the adrenal. Finally, the isolation of Δ^5-pregnene-3β,21-diol-20-one from urine after ACTH administration reported by Dobriner and his group (1950) is specially interesting for several reasons. In the first place, this substance has the same ring A configuration of dehydroepiandrosterone, a 17-ketosteroid excreted in comparatively large amounts by/









XXIII

	R_1	R_2	R_3
I	3β-OH	11β-OH	17α-OH
II	3α-OH	11β-OH	17α-OH
III	3β-OH	11-Keto	17α-OH
IV	3-Keto	11-Keto	17α-OH
V	3β-OH	H_2	17α-OH
VI	3β-OH	11β-OH	H
VII	3α-OH	11β-OH	H
VIII	3β-OH	11-Keto	H

	R_1	R_2	R_3
IX	3α-OH	11β-OH	17α-OH
X	3α-OH	11-Keto	17α-OH
XI	3-Keto	11-Keto	17α-OH
XII	3α-OH	H_2	17α-OH
XIII	3α-OH	11β-OH	H
XIV	3α-OH	11-Keto	H
XV	3α-OH	H_2	H

	R_1	R_2	R_3
XVI	11β-OH	CH_3	17α-OH
XVII	11-Keto	CH_3	17α-OH
XVIII	11β-OH	C(=O)H	H
XIX	H_2	CH_3	17α-OH
XX	11β-OH	CH_3	H
XXI	11-Keto	CH_3	H
XXII	H_2	CH_3	H

FIG.I.

α-KETOLIC STEROIDS ISOLATED FROM THE ADRENAL GLAND AND THE URINE.

Table 1. α-Ketolic steroids isolated from the adrenal gland and the urine.

	Compounds	Other Names	Adrenal	Urine
I	Allopregnane-3β,11β,17α,21-tetrol-20-one	Reichstein's V	+	+?
II	Allopregnane-3α,11β,17α,21-tetrol,20-one	Reichstein's C	+	-
III	Allopregnane-3β,17α,21-triol,11,20-dione	Reichstein's D	+	+?
IV	Allopregnane-17α,21-diol-3,11,20-trione	-	-	+?
V	Allopregnane-3β,17α,21-triol-20-one	Reichstein's P	+	+?
VI	Allopregnane-3β-11β,21-triol-20-one	Reichstein's R	+	-
VII	Allopregnane-3α,11β,21-triol-20-one	-	-	+
VIII	Allopregnane-3β,21-diol-11,20-dione	Reichstein's N	+	-
IX	Pregnane-3α,11β,17α,21-tetrol-20-one	Tetrahydro-F; TH-F	-	+
X	Pregnane-3α,17α,21-triol-11,20-dione	Tetrahydro-E; TH-E	-	+
XI	Pregnane-17α,21-diol-3,11,20-dione	Dihydro-E;DH-E	-	+
XII	Pregnane-3α,17α,21-triol-20-one	Tetrahydro-S; TH-S	-	+
XIII	Pregnane-3α,11β,21-triol-20-one	Tetrahydro-B; TH-B	-	+
XIV	Pregnane-3α,21-diol-11,20-dione	Tetrahydro-A; TH-A	-	+
XV	Pregnane-3α,21-diol-20-one	Tetrahydro-DOC; TH-DOC	-	+
XVI	Δ^4-Pregnene-11β,17α,21-triol-3,20-dione	Cortisol,Hydrocortisone, Kendall's F	+	+
XVII	Δ^4-Pregnene-17α,21:diol-3,11,20-trione	Kendall's E	+	+
XVIII /				

	Compounds	Other Names	Adrenal	Urine
XVIII	Δ^4-Pregnene-11β,21-diol-3,20-dione-18-al	Aldosterone, Electrocortin	+	+
XIX	Δ^4-Pregnene-17α,21-diol-3,20-dione	Reichstein's S, 17-OH-11-DOC	+	-
XX	Δ^4-Pregnene-17β,21-diol-3,20-dione	Corticosterone, Kendall's B	+	+
XXI	Δ^4-Pregnene-21-ol-3,11,20-trione	11-Dehydro-corticosterone, Kendall's A	+	-
XXII	Δ^4-Pregnene-21-ol-3,20-dione	11-Desoxy-corticosterone, DOC	+	-
XXIII	Δ^5-Pregnene-3β,21-diol-20-one	-	-	+

by normal people. Secondly, other steroids with the same grouping have been isolated from the urine of patients with adrenal carcinoma in fairly large quantities. The third reason is that pregnenolone, another Δ^5-3β-hydroxysteroid, appears to be a very important intermediate in the biosynthesis of the corticosteroids (Lieberman and Teich, 1953; Hechter and Pincus, 1954). We shall discuss later this group of steroids in more detail.

So far we have tabulated steroids which have been found sometimes in very small amounts, in abnormal cases, or after the administration of certain hormones. We shall proceed a step further trying to give a picture of the quantitative excretion of α-ketolic steroids by normal human beings.

Table 2 (p. 7) shows that tetrahydrocortisone is by far the most important urinary corticosteroid quantitatively speaking. Burstein, Savard and Dorfman (1953) found that the administration of hydrocortisone acetate to a male patient suffering from slight peripheral scleroderma was followed by the recovery in urine of increased amounts of compounds F and E and their/

Table 2.

REFERENCES	μg./24 hours				OBSERVATIONS
	E	F	TH-E	TH-F	
Baggett, Kinsella and Doisy (1953)	-	-	4,600	-	After β-glucuronidase; average of 14 men
De Courcy, Bush, Gray and Lunnon (1953)	91.5	34.5	-	-	After β-glucuronidase; average of 10 men
Cope and Hurlock (1954)	150	98	1,600	465	Hydrolysis cf. Bayliss (1952); average of 10 men

their tetrahydro derivatives. This is a proof of the conversion of cortisol to cortisone. As cortisol is the major corticoid in human blood (Bush and Sandberg, 1953; Morris and Williams, 1955) it follows that the estimation of these four compounds in urine can be a useful tool in the assessment of adrenal cortical function as far as the metabolism of cortisol is concerned (Cope and Hurlock, 1954).

Cortisol is also the major component of hog adrenal extracts (see Table 3, p. 9) and apparently of human adrenal glands (Hudson and Lombardo, 1955). All the evidence tends to indicate that cortisone is not present in human blood in detectable amounts by the methods now in existence. On the other hand, corticosterone is present in amounts ranging from one-half to one-tenth of those of cortisol. It is noteworthy that although the transformation of corticosterone into dehydrocorticosterone and their tetrahydro-derivatives has been shown <u>in vivo</u>, these substances have been detected in urine only after the administration of the parent compound (Richardson, Touchstone and Dohan, 1954).

The position may be summarized thus:-
Of the six active compounds isolated from the adrenal/

Table 3.

Amounts of active compounds found in beef adrenal (Fieser and Fieser, 1949) and in hog adrenal (Dobriner et al., 1954) in μg./kg.

Compounds	Beef	Hog[x]
Hydrocortisone	80	1,900
Cortisone	1,100	1,320
Corticosterone	750	440
Dehydrocorticosterone	735	1,100
17-OH-DOC	13	880
Desoxycorticosterone	27	-

x Wettstein and Anner (1954) have reported 9,000 μg. of corticosterone, 7,000 μg. of hydrocortisone and 3,500 μg. of cortisone per kg. of hog adrenal gland. Their figure for aldosterone is 40-95 μg./kg.

adrenal cortex, excluding aldosterone, desoxycorticosterone and its 17-hydroxylated derivative are not probably hormones but may play a role in the biogenesis of the other four. Hydrocortisone and corticosterone are the major corticoids in human blood. Together with aldosterone they account for all the physiological properties of adrenal extracts regarding carbohydrate and mineral metabolism and can therefore be properly called cortical hormones. Only α-ketolic steroids derived from cortisol have been found in the urine of normal subjects without prior administration of cortical hormones, the absence of derivatives of corticosterone being remarkable, because its administration is followed by their presence in urine.

It is a well known fact that steroid metabolites are excreted as their conjugates with sulphuric or glucuronic acid, and perhaps with amino acids (Eades, Pollack and King, 1954). The isolation of these conjugates has proved to be a difficult step in the characterization of the urinary corticosteroids, and so a preliminary treatment of the urine to split the conjugates is necessary. Bayliss (1952) and Marrian (1951) have/

have discussed the conditions that this hydrolytic treatment of the urine must fulfil. The former has suggested a procedure comprising treatment with the enzyme β-glucuronidase, extraction, adjustment of the urine at pH 1 followed by an immediate extraction and another after standing for 24 hours at room temperature.

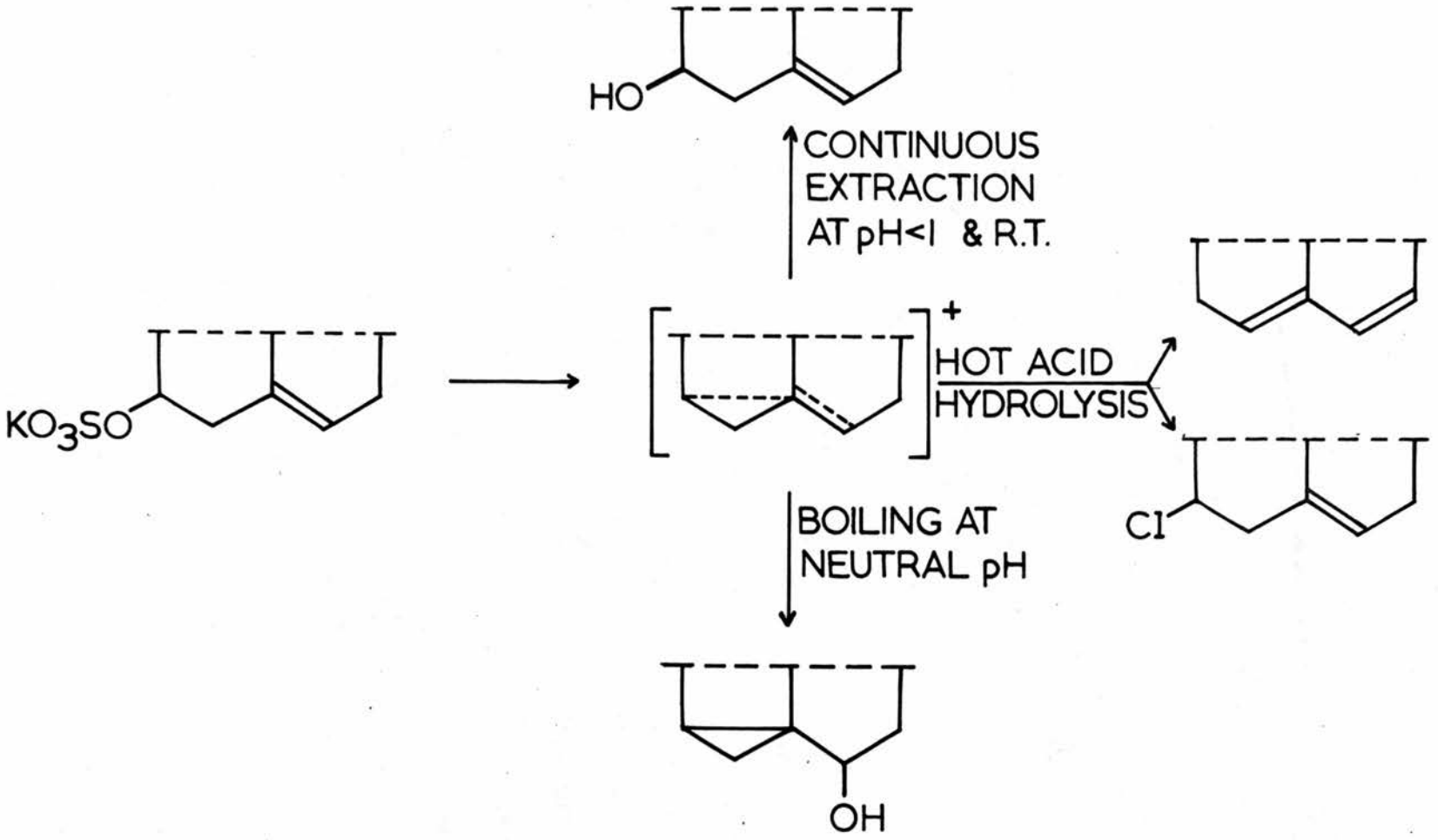
Although the flow-sheet outlined by Bayliss is probably the best for routine clinical purposes, it is doubtful if all the steroid conjugates are hydrolysed in this procedure without destruction. There is evidence for the existence of a type of conjugated formaldehydogenic material which can be extracted from acidified but not from neutral urine by chloroform, and is readily hydrolysed at pH 1 and room temperature, yielding free labile steroids under these conditions (Marrian, Paterson and Atherden, 1953; Paterson and Marrian, 1953b).

Speirs, Wragg, Bonner and Homburger (1951) have developed a bioassay based upon a decrease in the number of circulating eosinophils in adrenalectomized mice, a specific response to the administration of 11-oxycorticosteroids. With this method they made the observation that boiling of the urine of ACTH-treated patients, when

carried/

carried out at a neutral or slightly alkaline pH, produced an increase in the assay results. Boiling in an acid medium (pH 2) tended to destroy the corticoid activity. The increase only occurred in patients receiving ACTH and did not occur in patients receiving cortisone therapy. The boiling of neutral urine as a method of hydrolysis has been used by Dingemanse and Huis in't Veld (1952) who isolated i-androstan-6-ol-17-one from the urine of patients with virilizing tumours of the adrenal cortex. The original conjugate would be dehydroepiandrosterone sulphate according to evidence presented by Lieberman, Mond and Smyles (1954). The hydrolysis of this conjugate at pH 0.9 and room temperature for 24-48 hours accompanied by continuous extraction with ether yields only dehydroepiandrosterone with an excellent recovery. If, however, hot acid hydrolysis is employed the result is the formation of artifacts in a major proportion. These artifacts are predominantly 3,5-androstadien-17-one and 3-chloro- Δ^5-androsten-17-one. These transformations are shown in Fig. 2.

The present work started under the preceding considerations. The methods of separation of steroids in biological mixtures have/



HYDROLYSIS OF DEHYDROEPIANDROSTERONE SULPHATE. (FIG.2.)

have progressed to such extent that an investigation of the nature of the active material liberated by boiling was considered possible and very likely rewarding in view of the fact, as has already been pointed out, that this material was found only after ACTH administration and not after cortisone administration. This would suggest that the material is not a metabolic product of the cortical hormones, or cortisone at any rate, but perhaps an intermediate in their biosynthesis. When cholesterol-3-C^{14} is perfused through cow adrenal glands with ACTH in the perfusion medium, the increase in the production of corticoids with C^{14} is eighteen times that of non-ACTH-treated glands. If progesterone is substituted for cholesterol in this experiment, the corresponding increase is only one-fifth of the non-ACTH-treated glands. These results obtained by Stone and Hechter (1954) strongly indicate that the site of action of ACTH in corticosteroidogenesis is some reaction taking place in the conversion of cholesterol into progesterone.

Since the active compounds from the adrenal cortex all have an α-ketolic side-chain, a search for substances with this characteristic in extracts of boiled urine was the primary object of/

of this work. Its result has been the detection of a substance with reducing properties similar to those of α-ketolic steroids. This substance, whose properties have been studied in some detail, appears to be a hitherto undescribed corticosteroid. Its isolation and identification has not yet been achieved, but work in this direction is still proceeding in the hope that this finding may be correlated with the results reported by Speir et al. (1951).

Two other non-reducing substances have been isolated from these extracts of boiled urine. One of them, identified as 3β-Cl- Δ^5-androsten-17-one, is a well-known artifact occurring during acid hydrolysis of urine (Butenandt and Dannenbaum, 1934). The identification of the other was not possible due to the scarce amount of material available. There is evidence to show that this second compound may well be a 16-ketosteroid. Should this assumption be confirmed this would be the first instance of a compound of that kind isolated from human urine.

MATERIAL AND METHODS

Only a general description of the material and methods used in the present work will be given. The preparation of the urine extracts will be referred to in the first place, followed by a discussion of those physical and chemical methods of fractionation and analysis which have proved most useful in the course of this investigation. This section is not intended to be an exhaustive survey of the literature on these topics, but merely to contain the fundamentals of the methods, and the additions or modifications which were deemed convenient to make in order to adapt them to our conditions of work.

The Preparation of the Urine Extracts

The donors of urine were healthy male members of the staff of the Department of Biochemistry. The urine, whether brought to the laboratory in individual samples of 24 hours or being pooled during the day, was collected without preservatives and worked up immediately after the arrival. When this was not possible the urine was kept in the refrigerator for not longer than

48 hours after collection.

At the beginning of this work the urine was adjusted at pH 7 ± 0.2 with 0.1 N-NaOH. Later it was found that the processing of urine at its original pH when no less than pH 6 made no difference as regards the chromatographic pattern of the 'boiled urine' extract. The urine thus treated was then extracted with two volumes of chloroform and the extract washed twice with 1/5 of its volume of 0.1 N-NaOH, twice with 1/5 volume of distilled water, dried with 2% (w/v) of anhydrous sodium sulphate, filtered, and evaporated on a water bath at about 45°C. with the aid of a water pump. The residue was the crude 'unboiled urine' extract. The urine was then boiled for 30 minutes, cooled down under running water and extracted in an identical manner. This was the 'boiled urine' extract. The emulsions often found in extractions with chloroform were broken by filtration under reduced pressure or by centrifugation.

In the later isolation work, in which large volumes of urine were dealt with, the preceding procedure was uneconomic and time-consuming. Therefore, the urine was boiled without previous extraction, and afterwards was extracted with

4 /

4 x 0.1 volumes of chloroform, centrifuging after each extraction, and reintegrating the upper layer to the separating funnel for the next extraction. No important difference in the chromatographic pattern was seen in urines thus treated as compared with those on which the former procedure was used. No doubt, this extraction is still incomplete. Mr Fotherby of this Department (unpublished data) found that re-boiling of the urine followed by another extraction still liberated further amounts of formaldehydogenic substances, but from a practical point of view it was not considered necessary at this stage to extract exhaustively the urine until quantitative recovery of all the material was attained.

Chloroform and other solvents employed in this work were usually purified and redistilled in the laboratory following the directions of Weissberger and Proskauer (1935).

Physical/

Physical Methods of Fractionation

Solvent Partition. The crude extract prepared as described above was too impure to be applied to the starting line of the chromatograms. Mason, Myers and Kendall (1936) used successfully an elaborate method of solvent partition for the isolation of hormones from extracts of the adrenal cortex.

A very simple solvent partition between hexane and 70% aqueous methanol proved very useful in the present work. The reducing material was concentrated five times, and no reducing material was detected in the discarded phases. The procedure is as follows: The crude residue left after evaporating the chloroform extract of a 24 hr. sample of urine weighing 25 mg. or less was dissolved in 10 ml. of 70% aqueous methanol, and transferred to a small separating funnel. The flask was washed with a further 10 ml. of aqueous methanol and with two portions of 10 ml. of hexane, all the washings being transferred to the funnel. After shaking, the lower layer was drawn off, and another 20 ml. more of aqueous methanol added into the funnel to replace it. After this second, shaking/

shaking, the two methanolic fractions were collected together and evaporated under reduced pressure until the volume was about 10 ml., and then transferred to a funnel with the aid of 10 ml. of water. This aqueous solution was extracted with 50 ml. ether, the extract dried with a pinch of anhydrous sodium sulphate, filtered and evaporated to dryness.

Paper Chromatography. The application of this elegant method developed by Consden, Gordon and Martin (1944) to the separation of steroid mixtures has met with difficulties well emphasized by Bush (1954). Steroids have rather large, relatively flat molecules and very often are extremely sensitive to light, oxidizing agents, acids and bases, heat and strong adsorbents. Most of them are neutral, thus excluding ion exchange chromatography and the use of buffers as eluents. The partition ratio of the steroids favours the less polar phase in the common systems. The sheer number of steroid metabolites present in urine is in itself a limiting factor.

In spite of all these difficulties, many techniques exist nowadays which have already promoted our knowledge of the biosynthesis, and metabolic/

metabolic reactions of the steroid hormones (Bush, 1954; Savard, 1954; Haines and Karnemaat, 1954; Zaffaroni, 1953).

At first the chromatography involved the previous formation of the Girard's hydrazones of the ketosteroids (Burton, Zaffaroni and Keutmann, 1948 and 1949). Later a less cumbersome and more efficient method was developed by the same workers (Burton et al., 1951a and 1951b). The essential feature of their procedure is the impregnation of the filter paper with a non-volatile organic solvent as stationary phase, before its introduction into the chromatographic chamber. The solvent front is allowed to over-run the bottom edge of the paper and the overflow is collected in a beaker to be rechromatographed in a slower system. The R_F cannot thus be calculated, but the authors use the relative mobilities of the steroids to a fastmoving compound which receives a value of 1.

The previous impregnation of the paper is somewhat cumbersome and implies an additional difficulty in drying the chromatogram after development. Bush (1952) has proposed a method in which this difficulty is obviated by using volatile/

volatile solvents for both phases and carrying out the equilibration at 37°C. The stationary phase is aqueous methanol saturated with the mixture of organic solvents (benzene, ligroin, toluene, etc.) used as mobile phase.

The comparative usefulness of the methods of Zaffaroni and Bush has been discussed ardently by people in favour of one or the other method. It is a matter of personal choice and available facilities to decide which technique is most suited to a particular problem. Bush (1954) has summarized the advantages and disadvantages of both methods. It is probably true to say that Bush's method is better as an analytical technique, whereas Zaffaroni's method can handle relatively larger amounts of very crude extracts, and so, is better for preparative purposes.

Perhaps the greatest limitation of Bush's method comes from the need of a chromatographic room at 37°C. Schwarz (1953) and Alcock and Cannell (1954) have made apparatus designed to reduce the space required for each chromatographic sheet and to permit the introduction of this 'chromatocoil' - as Schwarz's device is named - into an ordinary incubator.

By/

By proper modification in the composition of the solvents, choice of the adequate quality of filter paper, and suitable time of equilibration of the paper with the vapours of the solvent, it is possible to adapt Bush's method so that it gives good results at room temperature. The amount of material that can be applied to the starting line is, however, considerably reduced. In our experience no more than 100 μg. per spot should be used when dealing with mixtures. This limitation is not of too great importance because very sensitive methods of detection are available which permit the location on the paper of 1 μg. of steroid or less in some cases.

This brings us to the question of the reagents or techniques used to find out the position of the steroids in chromatograms and, if possible, to reveal their identity. The spraying of the carefully dried paper with 10% NaOH in 60% aqueous methanol (Bush, 1952) or in water (Bush, 1954), followed by gentle heating at about 80°C., and inspection in the ultraviolet light of a mercury lamp provided with an adequate filter, shows the Δ^4-3-ketosteroids as yellow fluorescent spots. As little as 0.25 μg. may be/

be detected using photographic detection. In our experience, without the aid of photography, 10 μg. of steroid could be detected in a spot of 3 cm^2., but not smaller amounts. As there are in the urine extracts many other fluorescent spots, some of the yellow ones being very constant, it is necessary to examine the paper in the ultraviolet light before the spray.

Steroids such as the Δ^4-3-ketosteroids with a ketonic group conjugated with a double bond (α,β-unsaturated ketones) absorb maximally near 240 mμ. Use of this property is made in visualizing the opaque spots of these steroids when a fluorescent screen is interposed between the ultraviolet light source (a mercury lamp emitting light at 2537 Å) and the paper. In this form another test can be applied to the same paper, or if desired the spots can be cut out and the steroid extracted from the paper with a suitable solvent. As a warning of the lack of specificity of this test we should mention that caffeine and other purines and pyrimidines also give absorption under these conditions. Caffeine runs in our chromatograms slightly slower than Reichstein's compound S and is usually present in urine extracts.

The/

The 17-ketosteroids can be recognized by their reaction with alkaline m-dinitrobenzene to give purple spots (Axelrod, 1953) different from the much less sensitive reaction that takes place when the same reagent is applied to 3- or 20-ketosteroids.

For the characterization of the α-ketol grouping use has been made of the substance known as 'blue tetrazolium' (B.D.H.), first used by Mader and Buck (1952) in a colorimetric method for reducing steroids. 'Blue tetrazolium' (BT) is the 3,3'-dianisole-bis-4,4'-(3,4-diphenyl)-tetrazolium chloride (Rutenburg, Gofstein and Seligman, 1950).

BT can be applied in the same way as triphenyltetrazolium chloride (TPTZ) was (Burton et al., 1951a), but it is much more sensitive, detecting as little as 1 μg. of α-ketolic steroids per cm^2. Hoffmann and Staudinger (1951) suggested a quantitative method based upon the elution of the coloured product called formazan of the reaction between the steroid and TPTZ. Cope and Hurlock (1954) have proposed a similar method substituting the BT for the TPTZ, although the eluted/

eluted colour only follows Beer's Law within a certain range. It should be noted that BT is not specific for reducing steroids, other reducing substances giving positive reactions as well. Within the field of the steroids, however, it seems fairly specific as has been shown by Zaffaroni (1953), although not without limitations.

Only methods of detection used systematically throughout the course of this investigation have been described in the preceding paragraphs. Details referring to other reactions can be found in the references quoted, specially in the review by Bush (1954).

We can proceed now to a description of the chromatographic method that we have used as a routine analytical method in this research. The method using volatile solvents seemed preferable with the facilities available and the object we had in mind. Thus several trials placing the chromatographic chamber into an incubator were initiated. The results were very disappointing because of uneven heating of the front and rear walls of the chamber with the result that the papergram placed at the front was nearly developed when that at the back showed the solvent front midway/

midway between the starting line and the lower edge of the paper. The R_F values of parallel series of standard steroids run in this way showed considerable disagreement.

A series of experiments was designed to achieve a satisfactory working method at about 18°C. To this end, the quality of the paper, the composition of the solvents, and the time for equilibration were modified until finally consistent R_F values were obtained for the standards.

The paper chosen was Whatman no.42. The large sheets of this slow paper were cut in four portions measuring 14.3 x 45.7 cm. each. The starting line was drawn at 11 cm. from one edge. The application of the material to the paper was carried out by repeated pipetting by means of an opsonic pipette with a capillary tip. The paper was held on a hot plate while 2-4 µl. portions of the solution were successively applied on to the starting line. The resulting spot had a diameter of 5-10 mm.

The standard solutions were made up of a known weight of steroid in 10 ml. of methanol. The concentration was usually 1 µg./µl. These solutions were kept in the refrigerator until required. The urine extracts were applied in a similar/

similar fashion after solution in the minimum amount of methanol, which never was less than 0.1 ml.

The chromatographic chambers were glass tanks of adequate size, placed in a room at 18°C ± 2°C. Two solvent systems were used. The first, made up of benzene:water:methanol (10:3:7; v/v); the second was Bush's system B_2 (Bush, 1952). In either case, the solvents were shaken and left to separate in the chromatographic room overnight after occasional shaking during the day. The lower phase, stationary phase, or methanolic phase was drawn off into the tank. Part of the upper phase, i.e., the mobile phase, was poured into a dish which was placed at the bottom of the tank. Sheets hanging from the lateral sides of the tank and dipping into each phase facilitated the filling of the tank with the vapours of both phases. The solvents were renewed approximately every month. Within each batch and with similar length of running the R_F values for a given steroid were reproducible within ± 0.02.

The paper was hung from a trough placed at the top of the tank and the lid firmly secured by means/

means of a piece of wood screwed at both sides of the tank. After equilibrating overnight the mobile phase was poured into the trough through a small hole in the lid. The running lasted about 4 hr. for Bush's system B_2 and 6 hr. for the system benzene: 70% aqueous methanol. In this time the solvent front was at about 27 cm. from the starting line. The paper was removed and dried by evaporation at room temperature, and then inspected at the ultraviolet light of a Hanovia lamp for the presence of fluorescent substances which were marked with a pencil. In the second half of our study a 'Chromatolite' ultraviolet lamp was available to us, thus making possible the location of α,β-unsaturated ketones by the absorption of light of 2537 Å.

After this inspection, either Bush's reaction with the methanolic soda or the BT reaction was carried out. We have already given details of the first. To perform the second 10 mg. of BT were dissolved in 10 ml. of water and 5 ml. of 10% NaOH added. This volume is enough to spray a sheet of the size used in this work. We have not found it useful to combine both reactions, a procedure apparently successful in the hands of Bush (1954)/

(1954). Therefore, when information provided by both these reactions was needed, duplicates were run in separate papers. Otherwise, the BT reaction was systematically used.

The yellow fluorescent spots in Bush's reaction fade rather quickly, but a permanent photographic record can be kept. The BT spots are very stable and a copy on transparent paper can be made for keeping a record of the chromatograms.

Countercurrent Distribution

The earliest record of the use of countercurrent distribution in the analysis of urinary steroids is a short note by Archibald and Stroh (1948) who detected several peaks with a system consisting of cyclohexane and 38% aqueous ethanol.

Engel, Slaunwhite, Carter and Nathanson (1950) separated oestrone, oestradiol-17β, and oestriol by a 24-transfers countercurrent distribution using 50% aqueous methanol as the upper layer and carbon tetrachloride as the lower. The same system was used by Diczfalusy (1953) in his work on the oestrogen content of the human placenta.

A very interesting attempt to apply countercurrent/

current methods to the study of the pattern of urinary corticosteroids is the recent paper by Talbot, Ulick, Koupreianow and Zygmuntowicz (1955). The separation is carried out in a ternary biphasic system consisting of water, iso-octane and s-butyl alcohol (4:3:2). The analysis of the fractions is based upon the formation of the thiosemicarbazones of the α,β-unsaturated ketonic groups and their high extinction coefficient at 302 mμ.

Useful information about the partition coefficients of some neutral steroids in a quaternary system made of ethyl acetate, cyclohexane, ethanol, and water, has been presented by Engel, Alexander, Carter, Elliott and Webster (1954). Using 150 transfers and the above solvents in the proportion 5:5:3:7, Engel, Carter and Fielding (1955) have succeeded - after paper chromatography of some of the fractions - in isolating tetrahydrocorticosterone, its alloisomer (5α), and tetrahydrodehydrocorticosterone from the urine of a patient with rheumatoid arthritis who was treated with 300 mg. of corticosterone per day for 8 days.

We have made use of countercurrent methods in our work with not very good results. Of several solvent/

solvent systems which were tried, one consisting of methanol, water, benzene and chloroform in the proportions 16:4:17:3, was chosen because the partition coefficients of 17α-hydroxy-11-desoxy-corticosterone and 11-dehydrocorticosterone in it were respectively 1.44 and 0.60, the partition coefficient being the ratio between amount in the upper (methanolic) layer and amount in the lower (benzenic) layer.

In one case a hand-operating machine with 45 glass tubes of the type described by Gregory and Craig (1951) was used, whereas in a second experiment only 11 transfers using separating funnels were carried out. In both cases the volumes of upper and lower phases were the same. The solvents were mixed in the proportions quoted, allowed to settle, and separated. In a typical experiment 400 ml. of methanol, 100 ml. of water, 425 ml. of benzene and 75 ml. of chloroform gave 560 ml. of the methanolic upper phase and 430 ml. of the benzenic power phase.

Although the results of these distributions will be given later in detail, it can be anticipated here that countercurrent distribution of urine extracts/

extracts seems less valuable than other procedures such as paper or column chromatography in the isolation of steroid metabolites. It should be noted that in the work previously quoted (Engel et al., 1955) paper chromatography was needed to achieve a complete resolution of the main fractions.

Column Chromatography

This technique has rendered very valuable services in the resolution of the steroid mixtures present in extracts from tissues and biological fluids. A useful survey of the more relevant work published on this subject has been presented by Bush (1954a).

Speaking in general terms, we can distinguish adsorption and partition methods. In the ideal case, the basic phenomenon in adsorption chromatography is the concentration of the material dissolved in an adequate solvent upon the surface of a finely divided solid, whereas in partition chromatography a distribution takes place between two liquid phases, one of them held by an inert solid. It is universally agreed that these are extreme cases, rarely seen in practice, for the water/

water content of the adsorbent may introduce a partition effect and, conversely, the solid holding the stationary phase in partition chromatography may not be so inert as theoretically defined. The distinction, however, seems useful because it facilitates the theoretical treatment of the process of separation in each case.

In both methods a sufficient volume of a solvent or mixture of solvents is allowed to percolate through the column containing the adsorbent or the inert solid plus the stationary phase, in order to draw down the solutes which will gradually appear in a definite order. The liquid flowing from the column is thus collected in fractions whose contents can be analysed and the results plotted graphically as quantity of material in each fraction versus volume of effluent or fraction number (liquid chromatogram).

The form of the curve obtained for a pure compound can be shown to approach the normal Gaussian curve of error in the case of partition chromatography (Martin and Synge, 1941), in which the distribution isotherm is a straight line, at any rate for a certain range of concentrations. This is not the case in adsorption chromatography because the distribution isotherm is a parabolic (Freundlich's /

(Freundlich's isotherm) or hyperbolic curve (Langmuir's isotherm), i.e. the amount of solute adsorbed by a given weight of adsorbent is not a linear function of the concentration (Tiselius, 1952). The adsorption is relatively greater for small concentrations than for large ones. This will cause the rear end of a band of a solute in the column to move more slowly than the front end, with the result that the curve is no longer symmetric and shows a diffuse tail which may interfere with adequate separation from the front end of the next solute.

If the elution, however, is carried out by a continuously modified solvent, commencing the elution with a solvent which will not elute the solutes and then continuously adding - using an external mixing vessel - a second solvent in which they are not adsorbed at all, the rear part of a band is forced to move faster than the front because a concentration gradient of the second solvent is established in the column, favouring the desorption process of the small amounts of solute 'tailing' behind. (William, 1954).

There have been many applications of this device/

device, which has received the name of 'gradient elution chromatography', to the separation of biological mixtures. The manner in which the second solvent is added to the first in the mixing chamber is a matter that deserves consideration. Three principal ways of doing it can be found in the literature:

i) Using a mixing chamber from which the solvent mixture goes to the chromatographic column at the same rate with which the second solvent - with greater eluting power - enters the mixing chamber from a reservoir (Williams, 1952).

ii) Using a dispositive which permits the rate of inflow of the second solvent into the mixing chamber to be independent from the rate of outflow of the solvent mixture (Lakshmanan and Lieberman, 1953).

iii. Using two different reservoirs containing the solvents and both connected between them and to the column. The reservoirs are shaped in such fashion that the proportion of each solvent in the mixture is a function of this shape when the level of liquids in the reservoirs descends by gravitational force or after application of pressure (Bock and Ling, 1954).

The least satisfactory of these three methods is the first; the second allows a great flexibility and is to be preferred for research purposes; the third entails some preliminary work until a satisfactory design for the reservoirs is found, but has the great advantage of giving the same pattern of composition with maximum reproducibility, and can be very useful in routine work.

These three techniques have been applied to the separation of corticosteroids. Heftmann and Johnson (1954) have published a procedure for the separation of adrenocortical steroids on columns of wet silicic acid, employing a gradient of the first type with petroleum ether and dichloromethane as solvents, after saturation with water. Lakshmanan and Lieberman (1954) have used the second method, using an alumina column,and benzene and dilute solutions of ethanol in benzene as solvents. Furthermore, they presented the more complete study published so far on the theoretical and analytical aspects of gradient elution chromatography. Finally, Morris and Williams (1955) have used the third device to separate blood corticoids on partition columns, the supporting phase being Hyflo-Supercel holding ethylene glycol as stationary phase. The mobile phase/

phase was a mixture of 25% (v/v) light petroleum (B.P. 80-100°) in toluene progressively richer in pure ethylene dichloride. The shape of the concentration curve for this solvent in the eluent mixture is determined exclusively by the design of the apparatus and can be determined by measurement of the toluene content of the fractions by its ultraviolet absorption at 269 mμ.

The shape of the concentration gradient can be calculated if the initial volume of the solvent in the mixing chamber and the rates of inflow and outflow are known. We shall present in some detail the deduction of a formula similar to that of Lakshmanan and Lieberman (1954) but more general.

Let us adopt the following symbols:

C = Concentration (v/v) of solvent P in the mixing chamber.

k = Concentration (v/v) of solvent P in the mixture, contained in the reservoir, which is added to the mixing chamber.

Vo = Initial volume of solvents in the mixing chamber.

A = Rate of flow from the reservoir into the mixing chamber.

B = Rate of flow from the mixing chamber into the column.

a = Vo/A.

b /

$\underline{b}$ = $(A-B)/A = 1 - B/A$.

$\underline{Co}$ = Initial concentration (v/v) of solvent $\underline{P}$ in the mixing chamber.

$\underline{t}$ = Time.

$\underline{P}$ = The solvent with more powerful eluting properties.

$\underline{Q}$ = The solvent with small eluting power.

The mixing chamber may contain pure solvent $\underline{Q}$ and the reservoir pure $\underline{P}$ at the beginning of the experiment or mixtures of $\underline{P}$ and $\underline{Q}$ may be placed in both containers provided that $k > Co$.

If $\underline{y}$ is the partial volume of $\underline{P}$ in the mixing chamber at a given moment and $\underline{v}$ is the total volume of the mixture $\underline{P} + \underline{Q}$ in the mixing chamber at this moment, it follows that:

$$C = y/v \qquad (1)$$

After an infinitesimal lapse of time $\underline{dt}$, the concentration will have increased by $\underline{dC}$. Thus:

$$C + dC = (y + Akdt - BCdt)/[v + (A-B)dt] \qquad (2)$$

Subtracting (1) from (2)

$$dC = (k - C)Adt/[v + (A - B)dt] \qquad (3)$$

Also,

$$v + (A - B)dt = Vo + (A - B)t \qquad (4)$$

Substituting this value in equation (3), dividing numerator and denominator of the fraction by/

by A, and making use of the conventions given above,

$$\frac{dC}{k - C} = \frac{dt}{a + bt} \qquad (5)$$

The integration of this differential equation gives:

$$-\log(k - C) = \frac{1}{b}\log(a + bt) + K \qquad (6)$$

In which, K is a constant whose value can be obtained from (6) when t = 0:

$$K = -\log(k - Co)a^{1/b} \qquad (7)$$

Substituting the value of K in (6), we arrive at:

$$k - C = (k - Co)\left[\frac{a}{a + bt}\right]^{1/b} \qquad (8)$$

And finally,

$$C = K\left[1 - \left(\frac{a}{a + bt}\right)^{1/b}\right] + Co\left(\frac{a}{a + bt}\right)^{1/b} \qquad (9)$$

Equation (9) shows how C is a function of t. The first derivative of this equation with respect to time will give us the value of the eluant concentration gradient, dC/dt, and the second derivative will be the expression of the variation of this concentration gradient with time/

time, i.e. whether the concentration gradient decreases, increases or remains the same throughout the experiment. It will also allow us to predict the form of the curve corresponding to formula (9). These considerations are extremely important, for Lakshmanan and Lieberman (1954) have shown that the best results are obtained in gradient elution chromatography when the concentration gradient increases continuously, condition fulfilled when the second derivative is positive, the concentration curve thus being concave upwards.

The first derivative is:

$$dC/dt = C' = (k - Co)a^{1/b} \Big/ (a + bt)^{1/b + 1} \quad (10)$$

Only if C' is positive there will be a positive concentration gradient, i.e., a continuous increase in the concentration of the solvent F in the mixing chamber. This happens when $k > Co$ and $Vo/t \rightarrow B - A$.

The second derivative is:

$$dC'/dt = C'' = (Co - k)(b + 1)a^{1/b} \Big/ (a + bt)^{1/b + 2} \quad (11)$$

The following three cases may occur:

i) $b < -1$; this happens if $B > 2A$; then $C'' > 0$
ii) $b = 0$; this happens if $B = 2A$; then $C'' = 0$
iii) $b > -1$; this happens if $B < 2A$; then $C'' < 0$

Only/

Only in case i) the concentration gradient, dC/dt, will increase continuously and the concentration curve will be concave upwards, as is sought. In case ii) the concentration curve is a straight line, i.e., the concentration gradient remains constant. In case iii) the concentration gradient will decrease along the experiment and the concentration curve will be convex upwards.

The general formula (9) becomes simpler in special cases. If the liquid initially present in the mixing chamber is pure Q (Co = 0), equation (9) adopts the form:

$$C = k\left[1 - \left(\frac{a}{a + bt}\right)^{1/b}\right] \qquad (12)$$

If, besides, the liquid in the reservoir is pure P (k = 1), we can write:

$$C = 1 - \left(\frac{a}{a + bt}\right)^{1/b} \qquad (13)$$

When k = 1, but Co ≠ 0, the equation is:

$$C = 1 + (Co - 1)\left[\frac{a}{a + bt}\right]^{1/b} \qquad (14)$$

If the concentration gradient is constant (B = 2A), equation (9) becomes:

$$C = kt/a + Co(1 - t/a) \qquad (15)$$

If/

FIG.3. THEORETICAL GRADIENT ELUTION CURVES.

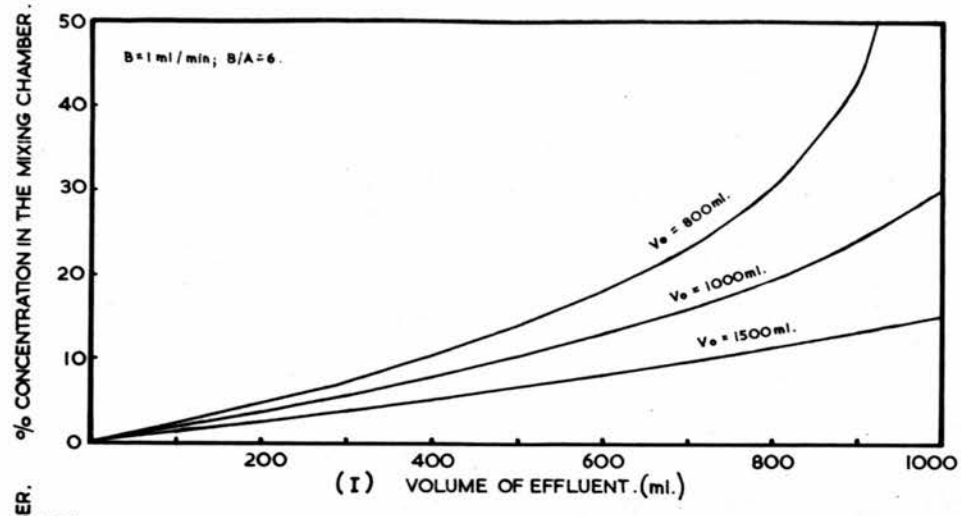
THE CONCENTRATION (V/V) IS THAT OF THE MORE POLAR SOLVENT ADDED TO THE MIXING CHAMBER. (see text).

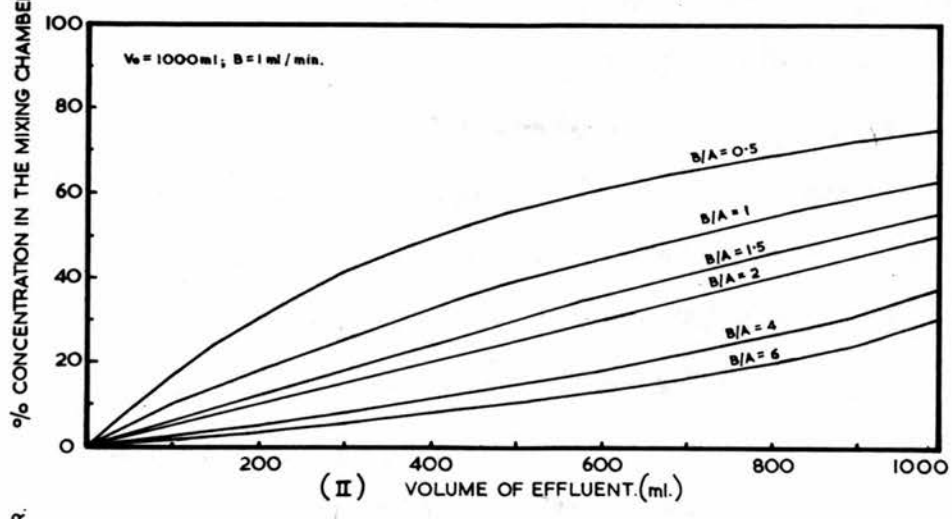
THE RATE OF WITHDRAWAL (B) FROM THE MIXING CHAMBER IS CONSTANT.

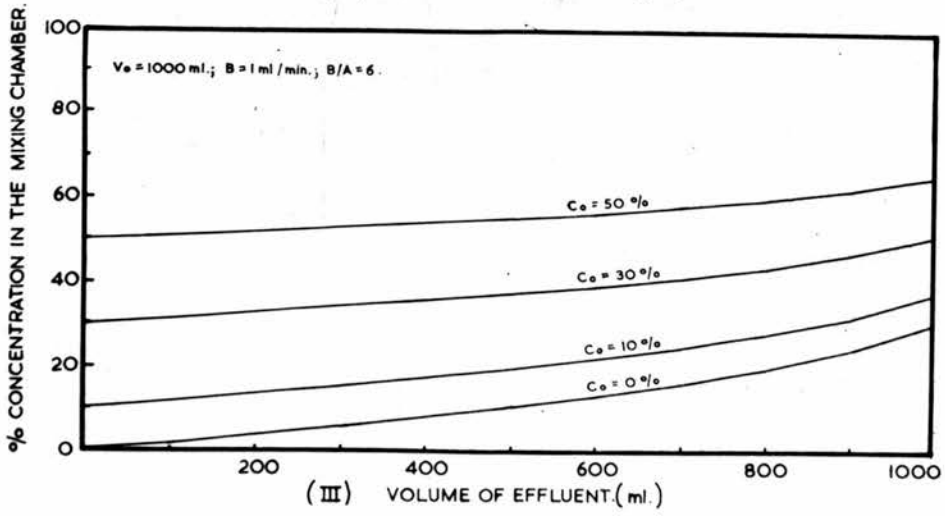
(I) EFFECT OF VARYING THE INITIAL VOLUME (V_o) IN THE MIXING CHAMBER.

(II) EFFECT OF VARYING THE RATE OF ADDITION (A) OF SOLVENT TO THE MIXING CHAMBER.

(III) EFFECT OF VARYING THE INITIAL CONCENTRATION (C_o) OF THE MORE POLAR SOLVENT IN THE MIXING CHAMBER.







If the volume in the mixing chamber remains constant ($\underline{B} = \underline{A}$), the concentration gradient constantly decreases, for it is a particular case of (iii) shown above, and the concentration curve is of the exponential type:

$$C = \left[k(e^{t/a} - 1) + Co\right] \Big/ e^{t/a} \qquad (16)$$

Fig. 3 is a clear demonstration of how the concentration gradient can be varied by changing the initial volume in the mixing chamber, the ratio B/A, or the initial concentration of the active solvent in the mixing chamber. The effect of giving $\underline{k}$ (concentration of the active solvent in the reservoir) values other than 1 is not shown in Fig. 3. It is sufficient to change the scale of Fig. 3(a) and 3(b) substituting the value of $\underline{k}$ (in vol. per cent.) for the top value of 100, as can be easily deduced from equation (12). This does not apply to Fig.3(c) where $\underline{Co} \neq 0$, unless the reduction in scale is the same for $\underline{k}$ and $\underline{Co}$.

The method developed by Lakshmanan and Lieberman (1954) is thus of immense flexibility, any desired gradient being easily obtained by changes of one or several of the values, $\underline{Co}$, $\underline{k}$, $\underline{Vo}$, $\underline{B}$, and $\underline{A}$.

The/

The degree of resolution in gradient elution chromatography is not necessarily better for using long columns. The same authors have shown that there is an optimum height of the column in order to achieve the best possible resolution of a group of substances. Among their other findings, it is worth mentioning that a moderate degree of moisture in the alumina gives better resolution, especially of the weakly adsorbed solutes. The reproducibility of the results is secured by equilibrating the alumina with an atmosphere of constant humidity supplied by a saturated solution of $NaBr.2H_2O$. Similar facts have been reported by Wolfrom, Thompson, Galkowski and Quinn (1952) about magnesium silicate.

Gradient elution has been used in this work with both partition and adsorption columns. The automatic fraction cutter available to us was unfortunately very inaccurate, and so this advantage of gradient elution chromatography - the automatic operation - was lost. It was decided to obtain the gradient by manual operation. The column was fed with small volumes taken from a flask to which the more polar or active solvent was/

was added. For instance, for each 10 ml. taken from the flask onto the column, 2 ml. of polar solvent were pipetted into the flask and the contents mixed by rotation. It should be noted that this procedure is, properly speaking, a stepwise method of increasing the concentration in the eluting mixture, although the difference with the described continuous gradient is insignificant provided that the volume of the fractions into which the eluate is divided is not greater than that placed at each step on top of the column.

The experimental details about the operation of these columns will be given later. It will now be sufficient to add that the partition method was that of Haines and Karnemaat (1954) and the adsorption method that of Lakshmanan and Lieberman (1954) but for the adoption of magnesium silicate instead of alumina. The magnesium silicate (B.D.H. magnesium trisilicate for chromatographic purposes) was moderately activated by leaving it overnight in an oven at 110°C.

Chemical Methods of Fractionation

Girard Separation

In the search for suitable reagents for the ketonic compounds existing in natural mixtures, Girard and Sandulesco (1936) found that the hydrazides of quaternary ammonium derivatives react easily in acid medium with ketones and aldehydes giving the corresponding hydrazones, which are very soluble in water. Aldehydes form very stable hydrazones, from which the original compounds cannot be regenerated even by strong mineral acid hydrolysis at the boiling point. The hydrazones obtained from the ketones are on the contrary easily cleaved at room temperature by dilute acids. In outline, their method consisted in refluxing for 30-60 min. the hydrazide (Reagent T) and the crude material dissolved in ethanol acidified with acetic acid; cooling and partially neutralizing to prevent spontaneous hydrolysis of the hydrazones; removal of the non-ketonic material by extraction with an organic solvent; acidification to split the hydrazones; and extraction to recover the separated ketones. The procedure proved to be useful in the commercial preparation of oestrone.

The/

The nature of other chemical groups in the neighbourhood of the carbonyl group modifies the reactivity of the compound with the hydrazide and the facility with which the cleavage of the resultant hydrazone can be achieved. Reichstein (1936) made use of this property in a method of fractionation which he applied successfully to the resolution of adrenocortical ketosteroids.

Sprechler (1950) has reviewed some of the modifications introduced in the Girard separation by different workers and has studied experimentally the various steps in it. His results confirm that the hydrazone formation proceeds with excellent yields by leaving the reaction mixture overnight at room temperature, and that quantitative hydrolysis is ensured acidifying at pH just below.1.0 and extracting two hours later.

The procedure followed as a routine in this work is very similar to one used by Schneider (1950) for the isolation of adrenocortical compounds from human urine and is given as a flow-sheet in Fig. 4. For more than 10 mg. of ketosteroids the amount of the reagents should be increased proportionately.

Digitonin/

Fig. 4. Girard Separation

1. Dissolve the crude fraction containing no more than 10 mg. of ketosteroids in 1 ml. of 50% (v/v) acetic acid in 90% (v/v) aqueous methanol. Add 100 mg. of Girard's reagent T and warm gently if necessary until complete solution. Leave the reaction mixture overnight at room temperature.

2. In the morning, add small pieces of ice and 10 ml. of ice-cold water. Transfer to a 50 ml. separating funnel. Add 3 ml. of ice-cold 2.5 N-NaOH. The flask is washed with 10 ml. of ice-cold water which are also transferred to the funnel.

3. Extract eight times with 10 ml. of ice-cold chloroform, allowing the separation of the two phases to take place each time. Combine the chloroform extracts.

AQUEOUS PHASE

4. Add 2 ml. of conc. HCl and extract immediately with 10 ml. of chloroform. Seven more extractions with 10 ml. portions of chloroform are carried out in the course of two hours.

Water phase discarded

Chloroform extracts combined and washed as in (5). The dry residue is the ketonic fraction.

CH_3Cl PHASE

5. Wash the combined chloroform extracts once with 15 ml. of 0.25 N-Na_2CO_3 and three times with 15 ml. of water.

Washings discarded

Chloroform extract dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue is the non-ketonic fraction.

of ketosteroids the amount of the reagents should be increased proportionately.

Digitonin Precipitation

Most 3β-hydroxysteroids present the property of precipitating the steroid sapogenin digitonin in alcoholic solutions. The complex thus formed is called a digitonide and can be split by warm pyridine, whereupon the steroid can be recovered by ether extraction. Extensive use has been made of this property in steroid chemistry for the fractionation of mixtures and the structural analysis of unknown compounds (Fieser and Fieser, 1949). The precipitation however is not absolutely specific, and some 3β-hydroxysteroids fail to form insoluble digitonides or may possibly not form digitonides at all. Conversely, quite a few compounds which are not 3β-hydroxysteroids, including non-steroidal substances, precipitate with digitonin. Haslam and Klyne (1953) have been able to enunciate some helpful generalizations. Those 3β-hydroxysteroids of the 5α-series (allo-pregnane and androstane) and of the Δ^5-unsaturated series form insoluble digitonides in much higher dilution than the corresponding steroids of the 5β /

5β-series (pregnane and etiocholane). Olefinic linkages at C-5, 6-7, C-8, or C-14 do not influence digitonin precipitability, except for Δ^5-androsten-3β-ol and some of the Δ^5-pregnene-3,20-diols. A ketonic group at C-17 or C-20 seems to reduce the precipitability. Hydroxyl groups in these positions are much more effective in this reduction.

In spite of these limitations the reaction is very useful. The procedure followed in the course of this work corresponds in general lines with that of Butt, Henly and Morris (1948). The main steps are the formation of the digitonide, its precipitation, the removal of the non-precipitated material ('alpha' fraction), the cleavage of the digitonide by pyridine, and the extraction of the so-called 'beta' fraction by ether. It is possible that some 3β-hydroxysteroids form soluble digitonides which are removed in the α-fraction by the ether added to the alcoholic medium. To recover them in the β-fraction the alcohol was removed by evaporation in vacuo after the formation of the digitonide. This modified procedure is described in Fig. 5. Valuable suggestions to the original procedure have been made by Haslam and Klyne (1952).

Fig. 5. Digitonide Formation

1. The material, containing no more than 0.5 mg. of 3β-hydroxysteroids, is transferred to a graduated glass-stoppered centrifuge tube and dried. Add 0.75 ml. of a 1% digitonin solution in 90% aqueous ethanol warmed at 37°C. Fit a microcondenser to the tube and reflux gently for 30 min. The solvent is removed carefully under reduced pressure.

2. 10 ml. of ether are added and the dry residue dispersed with the aid of a thin glass rod. Centrifuge for 5 min. at 2000 r.p.m. Decant the supernatant into a 30 ml. separating funnel. Repeat this step with three 5 ml. portions of ether.

SOLID RESIDUE

SUPERNATANT AND WASHINGS

4. The residue is dissolved in 0.25 ml. of pyridine, warmed to 60°C. for 3 min., cooled and 5 ml. of ether are added with stirring. Centrifuge, decant the supernatant into another 30 ml. funnel and repeat. The residue is further washed with 3 x 5 ml. of ether.

3. Wash the combined ether supernatants three times with 5 ml. portions of dist. water. Dry with a pinch of anhydrous sodium sulphate and evaporate. The residue is the

α-Fraction

5. The combined ether supernatants are washed twice with 5 ml. portions of 2N-sulphuric acid and three times with 5 ml. portions of dist. water. Dry with a pinch of anhydrous sodium sulphate and evaporate. The residue is the

β-Fraction

METHODS OF ANALYSIS

Spectral Analysis

Nearly all papers published in recent years dealing with studies on steroid metabolism report the use of one form of spectral analysis or another. It is not difficult to understand why this is so. Spectral analysis requires only small amounts of the substance which is investigated and very often it can be recovered for further analytical tests. The course of a metabolic reaction involving steroids can be easily followed when some of the products have a grouping with a characteristic spectral absorption. Dorfman (1953) has published an excellent review concerned with the ultraviolet absorption of nearly all steroids prepared or isolated up to 1951.

Zaffaroni (1950) developed a method of identification of the steroid hormones based on the determination of the absorption spectra of the coloured products resulting from the reaction between concentrated sulphuric acid and the steroids at room temperature. Certain generalizations seem possible. For instance, Δ^4-3-ketosteroids yield products with strong absorption at 280-290 mμ.; steroids/

steroids lacking hydroxyl groups usually present general absorption within the region examined, 220-600 mμ. Stereoisomers have very similar spectra differing only in the relative intensity of absorption of their maxima; the free steroids and their acetates produce identical spectra, as far as structural analysis is concerned (Zaffaroni, 1953). Bernstein and Lenhard (1953) have made a systematic study of the spectra of 220 steroids in concentrated sulphuric acid. They found that for all practical purposes Beer's Law is obeyed within the concentration range (<40-45 μg./ml.) examined. Some structural correlations have also been established by the same workers, Bernstein and Lenhard (1954); notably that selective absorption between 220-278 mμ. may correspond to the hydroxyl group, and to a lesser extent, to the isolated ketonic group. Axelrod (1953a,b) has also studied the spectra of some steroids in ordinary sulphuric acid, and in fuming sulphuric acid (15-16% of free SO_3) as well.

For the sake of completeness, infrared absorptiometry must be mentioned as capable of giving very useful information about molecular structure. It has been employed extensively

and/

and successfully in recent times in the identification of steroid metabolites (see Jones and Dobriner, 1949; Rosenkrantz, 1955).

The ultraviolet spectra described in this work were measured by means of a Unicam SP.500 photoelectric quartz spectrophotometer with silica cells of 1 cm. path. For the determination of spectra in sulphuric acid the directions given by Bernstein and Lenhard (1953) were followed. The time spent in reading a complete spectrum (220-600 mμ.) was about an hour.

Chemical Reactions:

Oxidation with blue tetrazolium

The tetrazolium salts are water-soluble compounds, usually colourless, which on reduction yield red or blue pigments insoluble in water known as formazans (Rutenburg, Gofstein, and Seligman, 1950). The oxidation-reduction potential of many of the tetrazolium salts overlaps those found in living systems, hence their wide use in histochemical techniques (Fox and Atkinson, 1950). Among these compounds, triphenyltetrazolium chloride (TPTZ, TTC, or TPT) was first used for the detection of the reducing primary α-ketol side/

side-chain of the adrenocortical hormones and their metabolites in paper chromatograms (Burton, Zaffaroni and Keutmann, 1951a). Later, Henly (1952) reported a colorimetric method for the estimation of corticosteroids using 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride, and Mader and Buck (1952) described a technique employing 'blue tetrazolium' (BT) whose composition has been given already. According to them, C_{21}-17α-hydroxy ketolic steroids produce less colour than C_{21}-17-deoxy steroids, and 11β-hydroxysteroids more than their 11-deoxy homologues. This clearly indicates that other groups in the steroid molecule may exert an influence on the chromogenicity of an α-ketolic steroid.

In this work, the methods developed by Chen, Wheeler and Tewell (1953) have been followed. These authors confirm that C_{21}-17α-hydroxysteroids are less chromogenic than C_{21}-17-desoxysteroids and state that α,β-unsaturated ketosteroids, as well as steroids with an α-ketol grouping in positions other than C-20 and C-21, react giving only 1/10 - 1/20 of the colour intensity corresponding to primary α-ketols, the time required for complete reaction being considerably longer. Zaffaroni/

Zaffaroni (1953) has tested ketolic steroids of the types 17-hydroxy-20-ketone, 11-hydroxy-12-ketone, 2-hydroxy-3-ketone, 2-hydroxy- Δ^4-3-ketone and 6-hydroxy- Δ^4-3-ketone with TPTZ and concludes that the reaction seems quite specific for a primary α-ketol group.

In a spot test on paper, oestradiol-17β-16-one gave a strong and immediate positive reaction when sprayed with BT solution. The intensity of the reaction was approximately identical with that given by an equal amount of Reichstein's compound S. Oestriol gave a negative reaction. Our experiment therefore indicates that the blue tetrazolium reaction is not specific for a primary α-ketol group.

Meyer, Hayano, Lindberg, Gut and Rodgers (1955) have also quite recently presented evidence against the supposed specificity of the blue tetrazolium reaction for primary α-ketols. In their view, TPT is more specific, although less sensitive. If BT is used, 6α-hydroxy- Δ^4-3-ketosteroids give a retarded reaction with development of approximately the same intensity of colour after 19 hours as that given by desoxy-corticosterone/

corticosterone after 20 minutes. Even more interesting, 19-hydroxy- Δ^4-androstene-3,17-dione developed in 45 minutes 60% of the intensity corresponding to the standard mentioned. Both types of compounds gave, on paper chromatograms, a retarded positive reaction with BT (4 min.) but did not react with TPT at the same concentrations.

It is then with these reservations in mind about the specificity of the method of Chen et al. (1953) that our quantitative estimations of α-ketolic steroids have to be interpreted.

Formaldehyde Estimations

The oxidation of C_{21}-steroids with either a ketolic (CH_2OH-CO-) or a glycolic (CH_2OH-CHOH-) side-chain by periodic acid causes the liberation of one equivalent of formaldehyde (Fieser, Fieser and Lieberman, 1944) which can be estimated by means of a colour reaction with chromotropic acid (MacFadyen, 1945), in the reaction mixture itself (Lowenstein, Corcoran and Page, 1946) or after being distilled from interfering chromogens (Daughaday, Jaffe and Williams, 1948).

The methods estimating formaldehyde produced by treating urine extracts with periodic acid/

acid may be as unspecific as those measuring the reducing properties, if not more. What renders them useful is the previous purification of the extracts, and the empirical correlation found between values of 'corticosteroid-like' material excreted in urine, as determined by these methods, and certain physiological and pathological situations, such as administration of adrenocortical hormones or ACTH, and different endocrine diseases, in which the adrenocortical function is known to be depressed or exalted.

The lack of specificity should nevertheless be borne in mind when interpreting results so obtained, particularly since substances which retain formaldehyde are present in urine extracts (Paterson and Marrian, 1953a). The method outlined by the last mentioned authors has been adhered to in the course of this work.

Other Chemical Reactions

The oxidation of C_{21}-17-hydroxysteroids with sodium bismuthate yields 17-ketosteroids if there is an oxygen atom at C-20, except in the case of 20-methyl-20-ketosteroids (Brooks and Norymberski, 1952), and constitutes a useful procedure/

procedure to characterize the corticosteroid side-chain, in combination with other tests. The 17-ketosteroids formed can be measured by the well known Zimmermann reaction (Callow, Callow and Emmens, 1938).

At a certain stage in this work it became necessary to know whether a substance eluted from paper chromatograms was a 3β-hydroxy-Δ^5-steroid or not. For this purpose a micro-method adapted from the procedure of Oppenauer (1941) for the oxidation of cholesterol to cholestenone was designed.

Other chemical tests occasionally used will be mentioned later in the description of the experiments.

RESULTS /

RESULTS

Study of 'Boiled Urine' Extracts by Means of Paper Chromatography

Preliminary Experiments

To explore the possibilities offered by paper chromatography in the study of the urinary corticosteroids a pilot experiment was set up, after having acquired some experience of Bush's technique (Bush, 1952) with pure steroids.

A 24 hr. sample of male urine was adjusted at pH 7.0 with 1 N-NaOH and made up to 2 litres with water. It was then extracted twice with 1.5 vol. of chloroform and the combined chloroform extracts washed twice with 0.2 vol. of 5% $NaHCO_3$ and twice with 0.2 vol. of distilled water. After drying with 2% (w/v) of anhydrous sodium sulphate and filtering, the extract was evaporated under reduced pressure on a water bath, the residue constituting the 'unboiled urine' fraction. The extracted urine was boiled for 30 min., cooled and re-extracted in the same fashion to obtain the/

the 'boiled urine' fraction. One-fifth of each fraction was transferred to a small test tube and dissolved in 0.1 ml. of methanol.

Bush's system B_5 was prepared in advance following his directions. The tank was placed in an incubator at 37°C. Whatman no.4 paper was used in sheets of 14 x 48 cm. drawing the starting line at 10 cm. from one of the edges. Two duplicate sheets were run and in each sheet three lanes were drawn perpendicular to the starting line. By repeated applications 0.025 ml. of each fraction were put on the starting line by means of an opsonic pipette with the tip drawn out. As standards, 50 μg. of cortisol and 50 μg. of Reichstein's compound S were applied to the paper.

The chromatograms were left to equilibrate overnight inside the tank. The development took about 200 min. The uneven temperature at the back and front walls of the tank caused an uneven development. In the sheet situated at the back the solvent front was 18.5 cm. from the starting line and the R_F values for compounds F and S were 0.48 and 0.85 respectively. The front of the tank/

tank was at a lower temperature than the back, and the sheet thus placed had the solvent front 28.5 cm. from the starting line, with R_F values for compounds F and S of 0.33 and 0.84 respectively. In spite of these technical imperfections the resolutions were satisfactory. The examination of the papers in the ultraviolet light of a Hanovia lamp revealed an array of blue, green, yellow and purple fluorescing spots in both the 'unboiled' and the 'boiled urine' extracts. After spraying with triphenyl tetrazolium chloride in aqueous methanolic soda (de Courcy, Bush, Gray and Lunnon, 1953), the familiar spots corresponding to tetrahydrocortisone, cortisol, and cortisone were detected in the 'unboiled urine' extract. Other two faint spots TPTZ positive, but not giving the yellow fluorescence at the ultraviolet examination, were present as well. In the 'boiled urine' extract two reducing spots slightly more 'polar' than compound S were clearly visible. None of them gave Bush's reaction for α,β-unsaturated ketones.

Another chromatogram of the same urine was run in Bush's system B_1 at 37°C. The existence of/

of a very strong TPTZ positive spot running slightly faster than compound S was confirmed and three other faint reducing spots were seen. The discrepancy in the position of the most important spot in both chromatograms - slower than compound S in B_5 and faster in B_1 - called for further investigation. In this second experiment the R_F values for compound S, DOC and the main spot were respectively 0.34, 0.81 and 0.45.

A second sample of urine was submitted twice to the extraction procedure outlined previously and then boiled for 30 min. and extracted again. Three fractions were thus obtained: U_1 and U_2 (two extractions before boiling) and B (extraction after boiling). The chromatograms of one-fifth of each extract run at 18°C. in Bush's system B_1 revealed the usual spots in U_1; there was a faint reducing spot with R_F = 0.07 in U_2; in B (the boiled urine extract) two reducing spots were seen. None of them gave yellow fluorescence in Bush's test with methanolic soda. One of them was weak and had an R_F = 0.14; the other was very strong, its R_F being 0.25. The corresponding values for the standards, compound S and DOC were 0.16 and 0.67 respectively. The result/

result of a similar chromatogram in Bush's system B_5 was disappointing for no reducing spot could be seen in the boiled urine extract.

Certain technical difficulties become apparent at this stage. In the first place, the spots on the starting line spread considerably during the equilibrium time. Secondly, the relative insensitivity of the TPTZ reaction made it necessary to use rather large volumes of urine extracts, the impurities of which were an important factor not only in the spreading just mentioned but also in the appearance of atypical reddish colours with TPTZ at the starting line and the solvent front, hampering the interpretation of the zones of the chromatogram with R_F = 0.00-0.20 and 0.80-1.00. Thirdly, the development of the chromatogram took place in a short time, about 2 hours, using Whatman no. 4 paper, which might have been insufficient for adequate distribution of the substances between the stationary and the mobile phase. All these factors added up to give inconsistent results, poor resolutions and too large spots.

To obviate these difficulties several measures were taken. Blue tetrazolium (BT) became/

became available to us and was used in aqueous alkaline solution instead of TPTZ, with a considerable gain in sensitivity which reduced the aliquot of the urine extract that had to be chromatographed. A very slow paper, Wtatman no. 42 was substituted for no. 4, and the temperature chosen for the chromatographic procedure was 18 ±2°C. Consistent figures for the R_F values of standard steroids were obtained using a modified Bush's system B_5, in which the concentration of methanol was increased to 70%, and Bush's system B_2. These two systems, working at 18°, give similar results, as far as R_F values are concerned, as systems B_5 (unmodified) and B_1 working at 34°C. Table 4 presents R_F values for some steroids in several systems. As reported by Bush (1952), compounds B and S cannot be separated, but no difficulty was experienced in resolving a mixture of compounds S and A. The time for the development to take place was about 6 hours for the modified Bush's B_5 and about 4 hours for Bush's B_2, the solvent front being at about 30 cm. from the starting line when the sheets were removed from the tanks at the end of these periods. With 10 µg. of steroid the resulting/

Table 4. R_F values of some adrenocortical steroids chromatographed at 18°C. on Whatman no.42. Data from Bush (1952) at 34°C. are included for comparison.

Systems		Compounds					
		F	E	B	S	A	DOC
Bush (1952)	System B_5	0.32	0.50	0.85	0.87	0.96	1.00
	System B_1	0.03	0.05	0.23	0.27	0.36	0.86
Benzene/ 70% Methanol at 18°C.	No.of expts.	18	17	-	18	-	-
	Range	0.17-0.24	0.34-0.41	-	0.60-0.72	-	-
	Mean	0.20	0.38	-	0.66	-	-
Bush's system B_2 at 18°C.	No.of expts.	-	-	2	28	9	23
	Range	-	-	0.34*-0.36	0.26-0.38	0.46-0.54	0.80-0.89
	Mean	-	-	0.35*	0.32	0.49	0.85

* The corresponding R_F values for compound S in these two chromatograms were 0.36 and 0.38. Therefore compound B is slightly more 'polar' than compound S, and not less, as might be inferred from a comparison of the averages in the Table. In neither of the two chromatograms was a mixture of compounds S and B resolved.

resulting spot had a size of the order of 2 cm. in length by 1.5 cm. in width. Finally the urine extracts were purified by means of the hexane/aqueous methanol partition described in the preceding section with a considerable improvement on the initial spreading of the spots.

These standard conditions were observed throughout the rest of this work, together with the procedures for detection already described.

Studies on the Urinary Excretion of Corticoids by Normal Men

When the chromatographic procedure was standardized, it became necessary to study a series of normal male urines in order to confirm the findings of the pilot experiments, namely, the existence of a considerable amount of BT-positive material in the 'boiled urine' extracts concentrated in the main in a zone corresponding to compound S. This study was, of course, indispensable before any serious attempt to isolate and identify the substance(s) responsible for the BT reaction could be undertaken.

Six healthy male members of the Departmental staff were the donors. The 24 hr. samy collected/

collected without preservatives, were adjusted to pH 7.0 immediately after the arrival at the laboratory. After one extraction with 2 vol. of chloroform the extracted urine was boiled for 30 min. and re-extracted with another 2 vol. of chloroform. The first chloroform extract was the 'unboiled urine' extract, the second was the 'boiled urine' extract. The first should contain the corticoids eliminated as the free compounds. The second should have corticoids excreted as conjugates partly or totally hydrolysable by boiling. It should be emphasized, however, that some degree of hydrolysis due to bacterial contamination may be responsible for the presence of a small part of the cortico-steroids in the first extract, while in the second extract there may be small amounts of free corti-coids due to incompleteness of the first extraction.

Both extracts were separately washed twice with one-fifth of their volume of 0.1 N-NaOH and twice with one fifth vol. of water, dried with 2% (w/v) of anhydrous sodium sulphate, filtered, and evaporated under reduced pressure on a water bath. These crude residues were purified by a partition between hexane and 70% aqueous methanol, as described in the chapter on 'Methods'. One-

fourth/

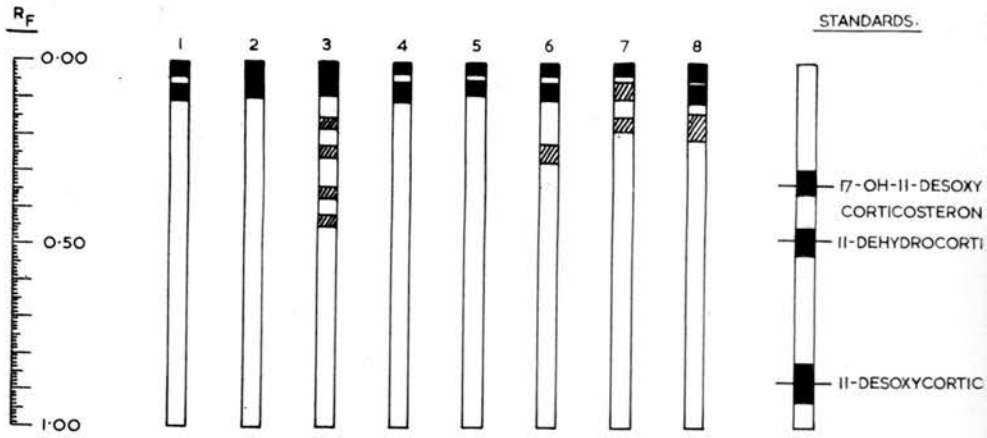
fourth of each extract was used to determine the formaldehyde liberated by periodic acid oxidation (Paterson and Marrian, 1953a). The remaining three-fourths of each extract were dissolved in 0.3 ml. of methanol.

Four chromatographic sheets were set up. Each contained 60 μl. of the two extracts and, in a central spot, the standards F, E, S, A and DOC in amounts ranging from 5-20 μg. Two of the papers were run in system B_2 and the other two in the system benzene-70% aqueous methanol, and then examined in the ultraviolet light and any fluorescent spot - specially yellow - marked with pencil. Of the two papers run in each solvent system, one was sprayed with BT and the other with methanolic soda (Bush, 1952).

The results are diagrammatically represented in Figs. 6-9. In these figures, the strips 1 to 6 correspond to six normal subjects, whereas 7 and 8 belong to a female patient before and after ACTH administration and will be discussed later.

The only strong BT-reducing spots in the unboiled urine that were regularly present occupied positions in the chromatograms consistent with the tentative identification as cortisol and/

FIG. 6.

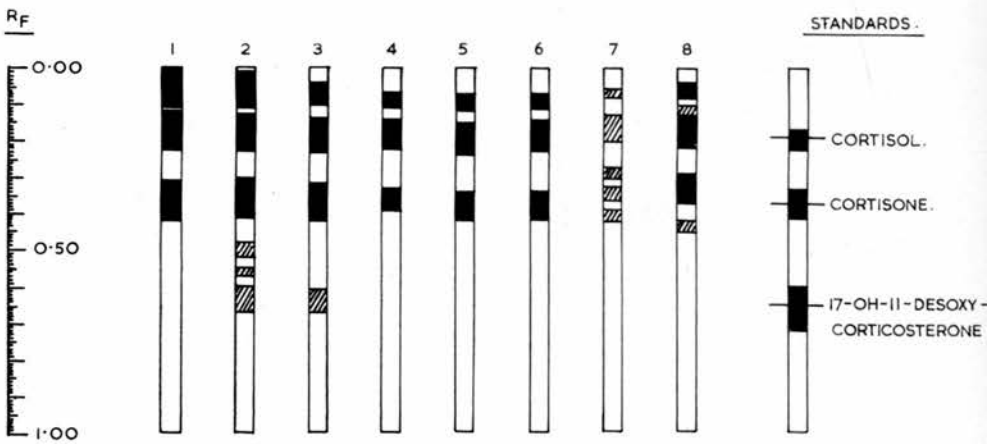


PAPERGRAMS OF UNBOILED-URINE EXTRACTS CHROMATOGRAPHED IN BUSH'S B_2 SYSTEM.

BLUE TETRAZOLIUM REDUCING SPOTS.

■ STRONG AND ▨ FAINT. (see text).

FIG. 7.

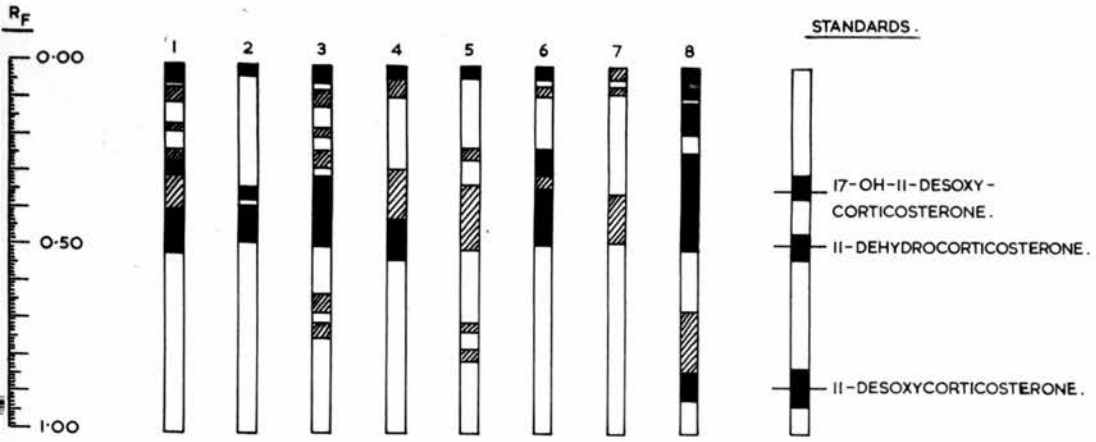


PAPERGRAMS OF UNBOILED-URINE EXTRACTS CHROMATOGRAPHED IN THE BENZENE / 70% AQ. METHANOL SYSTEM.

BLUE TETRAZOLIUM REDUCING SPOTS.

■ STRONG AND ▨ FAINT. (see text.)

FIG. 8.

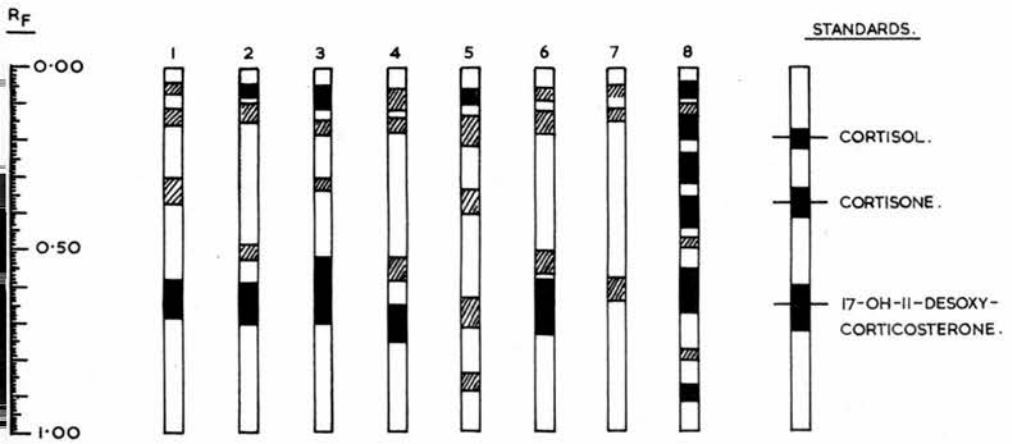


PAPERGRAMS OF BOILED-URINE EXTRACTS CHROMATOGRAPHED IN BUSH'S B_2 SYSTEM.

BLUE TETRAZOLIUM REDUCING SPOTS.

■ STRONG AND ▨ FAINT. (see text).

FIG. 9.



PAPERGRAMS OF BOILED-URINE EXTRACTS CHROMATOGRAPHED IN THE BENZENE/70% AQ. METHANOL SYSTEM.

BLUE TETRAZOLIUM REDUCING SPOTS.

■ STRONG AND ▨ FAINT. (see text).

and cortisone. The resolution of this material was not satisfactory using Bush's system B_2 because of its great 'polarity' (Fig. 6). In the other system (Fig. 7) the material was distributed in three major spots: two of them also gave a positive test with Bush's reagent - cortisol and cortisone - while the proximity of the third to the origin (R_F about 0.10), in a zone loaded with pigments, made it difficult to decide in each particular chromatogram whether the yellow fluorescent spots observed after spraying were the same as those BT-reducing spots observed in the duplicate chromatogram, although it is more likely that they were not. No reducing spots less 'polar' than compound E were seen with regularity.

The pattern of the 'boiled urine' extracts was more irregular. In all, in six cases there was a very 'polar' material in varying amounts remaining at the origin in system B_2 (Fig. 8) and moving with an approximate R_F of 0.08 in the other. There were also some faint spots with no regularity at all (see strips 3 and 5, Fig. 8). The largest proportion of the reducing material appeared in system B_2 as a band occupying approximately/

approximately the space between compound S and compound A, i.e., R_F = 0.25-0.50. The upper limit of this band always was a little above the upper limit of compound S spot. Although the resolution of this band into individual spots was never complete, a differentiation between a relatively faint spot slightly more 'polar' than compound S, and a very strong one rather less 'polar' could be made. The strip representing the standards has been drawn for each figure calculating the averages of the R_F values for the compounds run at the same time as the urine extracts, and the length for each spot represents the range of R_F values. This explains why in strip no.2 the spot with an R_F = 0.35 appears to be less 'polar' than compound S when in fact compound S had an R_F of 0.36 in the same chromatogram. The resolution of this main band of reducing material was less regular in the system benzene-70% aqueous methanol (Fig. 9), being clear-cut only in three cases (strips 2, 4 and 6).

It is interesting to compare the results of the chromatograms with the data obtained from the formaldehyde determinations presented in Table 5. Roughly speaking, the 'boiled urine' extract/

Table 5. Formaldehyde determinations on 'unboiled' and 'boiled urine' extracts in six normal men (μg. HCHO/24 hr.).

Case No.	'Unboiled'	'Boiled'
1	24.4	26.4
2	28.0	28.0
3	16.8	17.6
4	16.8	16.0
5	10.4	13.6
6	16.0	16.4
Average	19.6	18.7

extract contains as much formaldehydogenic substances (FSS) as the 'unboiled urine' extract. Furthermore, there is some correlation between the size and intensity of the reducing spots in the chromatograms with the amount of formaldehyde produced.

Strips nos. 7 and 8 show the effect of one day's ACTH administration (50 mg. every 6 hours) upon the elimination of urinary corticosteroids by a 62 years old female patient with heart disease. The control values of FSS for the 'unboiled' and 'boiled urine' extracts were 8.4 and 8.0 μg. per 24 hours respectively. After ACTH administration the corresponding values were 38.4 and 44.4 μg. per 24 hours. The chromatograms obtained after ACTH administration revealed a large number of reducing spots not present in the control urine or in the normal cases examined. The outstanding feature, however, is the increase in size and intensity of the main band above mentioned, which is clearly seen in Fig. 8.

Discussion/

Discussion

Very little will be said about the pattern of excretion reflected in the chromatograms of the 'unboiled urine' extracts. The position of the two less 'polar' compounds, identical with that of cortisol and cortisone, and the positive Bush's reaction (Bush, 1952) leave no doubt about their identity, especially since Schneider (1950, 1952) has isolated both compounds from untreated urine, as well as dihydro- and tetrahydrocortisone. It is likely that the more 'polar' reducing spot in the chromatograms of the 'unboiled urine is tetrahydrocortisone. Unfortunately the standard compound was not then available to us. De Courcy, Bush, Gray and Lunnon (1953) detected only cortisol and cortisone in the combined extracts obtained before and immediately after acidification to pH 1, but they used TPTZ as reagent, which is unanimously recognized as much less sensitive than BT, and they applied to the paper one-tenth to one-quarter of the sample, while in our case three-twentieths of the 24 hr. sample has been used consistently.

In the 'boiled urine' extract the

outstanding/

outstanding finding is the band of reducing material which spreads from just a little above compound S to the upper limit of compound A. The presence of this band cannot be attributed to incomplete extraction, as was shown by the preliminary experiment described before. There are other reducing spots sporadically present, besides a certain amount of material running down very little further from the starting line. Quantitatively speaking, it appears that the main reducing band constitutes the most important reducing material liberated by boiling, as far as can be judged from the visual inspection of the chromatograms.

The main band consists of two zones incompletely separated: one slightly more 'polar' than compound S, hereinafter called Y, and the other, much larger and more intense, running between compound S and compound A, hereinafter called X. The use of these letters does not imply that each zone contains only one reducing compound, but only that no further resolution could be obtained.

The positions of these spots and their failure/

failure to give a positive Bush's reaction indicate that they cannot be identified with any of the seven active principles of the adrenal cortex. From their running properties the assumption may be made that they have four oxygen atoms (like compounds S and A) if they are C_{21}-α-ketols. An examination of Table 1 and Fig. 1 discussed in the Introduction reveals that Reichstein's substances P, R, N, allotetrahydro-B and the tetrahydro derivatives of compounds S, B and A among the known adrenal and urinary corticosteroids have four oxygen atoms and do not give a positive Bush's reaction. The tetrahydro derivatives are much more 'polar' than their unsaturated precursors, and on this ground those of B and S can confidently be excluded. Reichstein's compounds P, R and N have not been isolated from urine, and since they are present in ox adrenal extracts in such small amounts, it is unlikely that they could be excreted in recognisable quantities. In view of these considerations it seemed that X and Y might be urinary corticoids as yet unidentified. Allotetrahydro-B and tetrahydro-A had not yet been isolated from urine at the time of these experiments/

experiments were performed.

An estimation of the amounts of X + Y can be made from the inspection of the spots compared with those of the standards. In terms of reducing steroid, about 0.1 mg. of both 'compounds' is eliminated per 24 hours. The average value for the formaldehyde liberated by the 'boiled urine' extracts (Table 5) was 18.7 µg./24 hours. Expressed in terms of compound S this figure is equivalent to 216 µg./24 hours. Thus, roughly speaking, half the formaldehydogenic material liberated by boiling is constituted by X + Y.

More accurate determinations have been made by Fotherby (unpublished data) in experiments parallelly conducted in our Department. Some of his results are included in Table 6. They also confirm that ACTH greatly stimulates the elimination of FSS in some conjugate form which can be split by boiling. X and Y are proportionately increased.

Table 6 /

Table 6. Formaldehydogenic substances (FSS) and reducing material present in 'boiled urine' extracts (μg./24 hr.).

Subject		X	Y	FSS[x]
Normal male		156.0	20.6	318.0
Normal male		158.0	51.5	332.0
Normal male		76.0	-	263.0
Male Patient	Before ACTH	150.0	56.0	277.0
	ACTH	467.0	155.0	1430.0
	After ACTH	120.0	78.0	505.0
Male Patient	Before ACTH	-	-	402.0
	ACTH	400.0	88.0	249.0?
	ACTH	336.0	144.0	288.0?
	ACTH	320.0	132.0	1108.0
	After ACTH	70.0	26.0	83.0

x FSS is expressed in terms of compound S.

Summary

a) In extracts of urine adjusted at pH 7.0 cortisol, cortisone, and a more 'polar' compound, presumably tetrahydrocortisone, have been detected by means of paper chromatographic methods which are a modification of those of Bush (1952).

b) Boiling of neutral urine previously extracted yields further amounts of formaldehydogenic substances and the chromatograms of these extracts show a band of reducing material incompletely resolved into two zones. One is small and runs slightly more slowly than compound S, whereas the larger runs between compound S and compound A.

c) None of these zones shows yellow fluorescence when viewed in ultraviolet light after being sprayed with methanolic soda (Bush's reaction for α,β-unsaturated ketones).

d) The amount of this reducing material is estimated to be within the range 0.1-0.2 mg./24 hours.

e) /

e) The administration of ACTH results in approximately a five-fold increase of the FSS eliminated, as well as in the amount of reducing material.

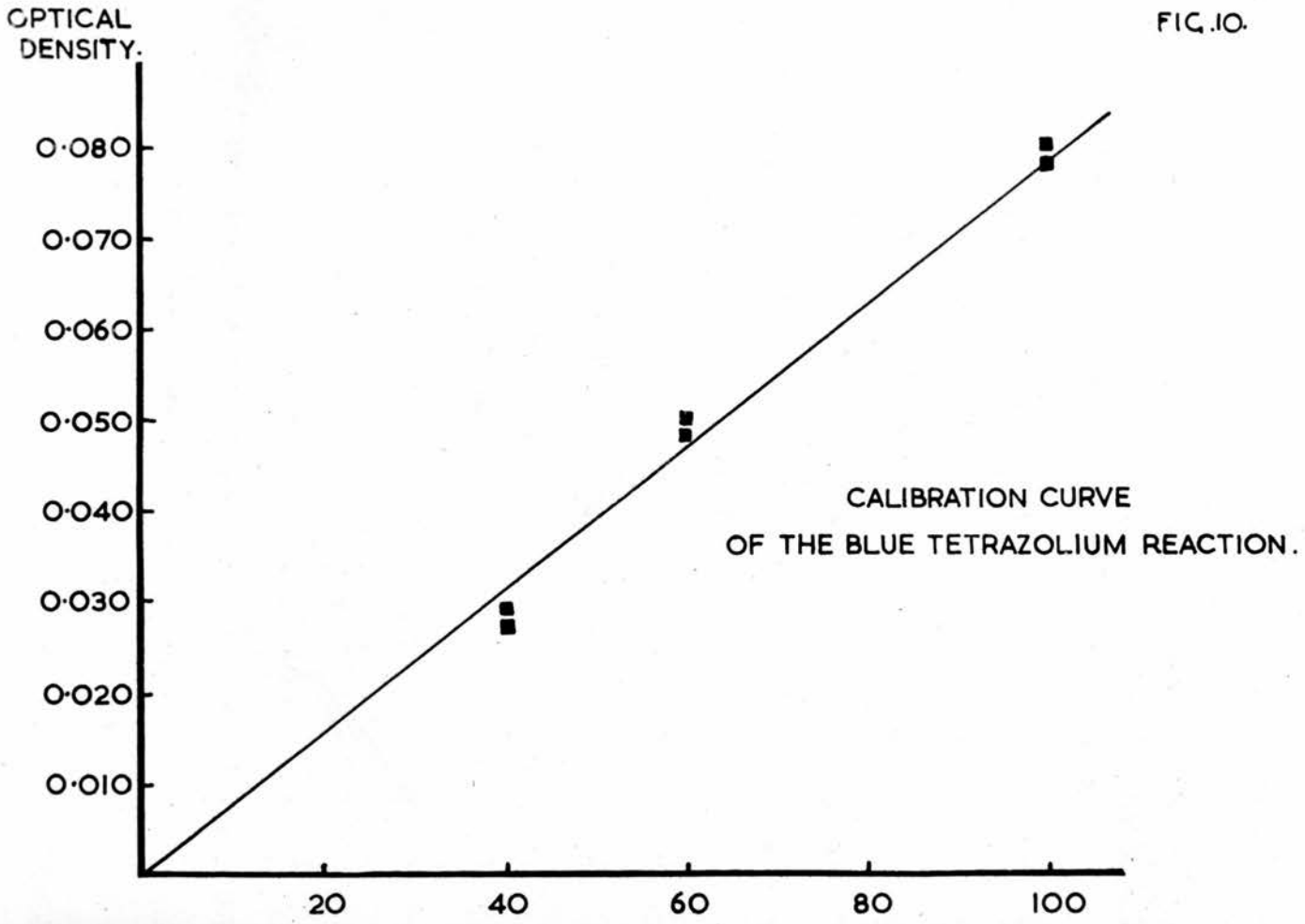
f) Certain considerations are made as to the possible nature of this reducing material.

Study of 'Boiled Urine' Extracts by Means of Countercurrent Distribution

Two experiments are reported here in which countercurrent methods have been applied to the study of the reducing material found in 'boiled urine' extracts. The first was designed as a pilot experiment to explore the possibilities that this method of separation could offer for the isolation of the reducing material in the pure state. The second was merely intended as a preliminary purification of this material in order to be able to apply other methods of isolation and to study the chemical and physical properties of the purified fractions.

To measure the amount of reducing material existing in each fraction the blue tetrazolium method proposed by Chen, Wheeler and Tewell (1953) using sodium hydroxide was used. As can be seen in Fig. 10 the colour developed does not depart from Beer's Law to any great extent. The use of calibration curves was regarded as unreliable because great differences in the optical densities for a given amount of standard were regularly obtained. Whenever this method was used known amounts/

FIG. 10.



CALIBRATION CURVE
OF THE BLUE TETRAZOLIUM REACTION.

amounts of cortisol were run through at the same time, and results, when not given in optical density values, represent cortisol equivalents.

Choice of Solvent System for the Countercurrent Experiments:

According to Gregory and Craig (1951) the most favourable conditions for the separation of two substances by means of countercurrent distribution take place when their partition ratios are reciprocals. These authors also show that the quality of the separation is a function of what they call 'separation factor'. This is simply the ratio of the larger partition ratio to the smaller. To obtain a given degree of separation, i.e., a given distance between the maxima corresponding to two substances in the distribution curve, it must be remembered that when the separation factor decreases the number of transfers has to be increased accordingly. Calculations from the formulae given by these authors shows that two substances having partition ratios of 1.56 and 0.64 can be separated by a 45-transfers countercurrent distribution to the extent of obtaining each one more than 93% pure, if all the material is recovered.

The/

The zone called X in the previous section is present between compounds S and A. It may reasonably be assumed that a solvent system in which the partition ratios of compound S and compound A were respectively 1.56 and 0.64 would provide very favourable conditions for the concentration of X in the middle of the distribution.

Accordingly, the following experimental determinations of partition ratios were carried out. Two aliquots of about 340 μg. of compound S each were evaporated to dryness in round-bottom flasks. A system of solvents was prepared beforehand equilibrating a mixture (v/v) of methanol, water, benzene and chloroform in the proportions 80:20:80:20. The upper layer was the methanolic phase. The dry residues were transferred from the flasks into small separating funnels with two portions of 20 ml. of the upper phase and two portions of 20 ml. of the lower phase, the mixture was vigorously shaken and the two layers collected separately, evaporated to dryness, and an aliquot taken for formaldehyde determination. A partition ratio of 1.74 (amount in upper layer/amount in lower layer) was the average value obtained from these/

these two samples.

In another similar experiment the system methanol, water, benzene and chloroform in the proportions (v/v) 80:20:85:15 was tried. The partition ratios for compounds S and A (upper layer/lower layer) in this system were 1.44 and 0.60 respectively. The experimental data of this and the preceding experiments are summarized in Table 7 (p.82). It must be emphasized that the recoveries were not always 100%. In the experiments with the second solvent system the recoveries for compound S averaged 73% and for compound A, 94%. The low recovery for compound S may be due to some decomposition of the standard solution used.

The last system was chosen for the two countercurrent distributions whose description follows.

First Countercurrent Distribution.

Twenty urine specimens of 24 hours each were extracted before and after boiling as described in the preceding section. All the crude 'unboiled urine' extracts were pooled together, and the same was done with the 'boiled urine' extracts/

Table 7. Distribution of compounds S and A in the systems:

I. Methanol, water, benzene and chloroform (80:20:80:20)

II. Methanol, water, benzene and chloroform (80:20:85:15)

Phase		Compound S(∝g.)		Compound A(μg.)	
I	Upper	206	218		
	"	230			
	Lower	105	125		
	"	145			
II	Upper	302	292	94	125
	"	282		156	
	Lower	204	203	168	211
	"	202		254	

extracts. These combined extracts were submitted to the hexane-70% aqueous methanol partition, the methanolic phase reduced to 1/4 of its volume by evaporation under reduced pressure, and the remaining liquid made up to the initial volume with water. This solution was extracted with ether and the ether extract washed with small volumes of N-HCl, N-NaOH and water, dried and evaporated to dryness. Aliquots of these residues were taken in duplicate for formaldehyde determinations. The experimental data are given in Table 8 (p. 84).

A hand-operated countercurrent apparatus with 45 glass units of the type described by Gregory and Craig (1952) was employed. Each unit held 25 ml. of each phase of the equilibrated system methanol, water, benzene, and chloroform (80:20:85:15) in which the aqueous methanol constitutes the travelling upper layer.

The ether residue was introduced into the apparatus dissolved in the first 25 ml. of upper layer. The time to perform 44 transfers was about 6 hours. Due to faulty calibration of the glass units a small fraction of the upper layer was not decanted to the next unit, thus reducing the actual volume of upper layer in equilibrium with the/

Table 8. Fractionation of the urine extracts of twenty 24-hours specimens totalling 27.6 l. for the first countercurrent distribution.

Fraction	mg.	mg./24 hr.	mg./l.
UNBOILED URINE:			
Crude extract	278.2	13.910	10.100
Hexane residue	36.6	1.830	1.325
Ether residue	19.5	0.975	0.705
FSS (as Compound S)	3.9	0.195	0.141
BOILED URINE:			
Crude extract	231.1	11.550	8.370
Hexane residue	38.5	1.925	1.395
Ether residue	51.1	2.550	1.855
FSS (as Compound S)	5.4	0.270	0.196
Reducing material[x] (as Compound F)	2.7	0.135	0.098

x Recovered after the distribution.

the lower layer in each glass unit. To overcome this cause of error additional small portions of upper layer were introduced into the apparatus at intervals up to a total of 50 ml.

At the end of the distribution the contents of the glass units were quantitatively transferred to test tubes with chloroform and methanol and evaporated to dryness. One-fifth of each fraction was taken to determine the reducing material by the blue tetrazolium method. The results of these determinations are given graphically in Fig. 11. The total reducing material recovered from the distribution ammounted to 2.7 mg. in terms of the standard used, cortisol.

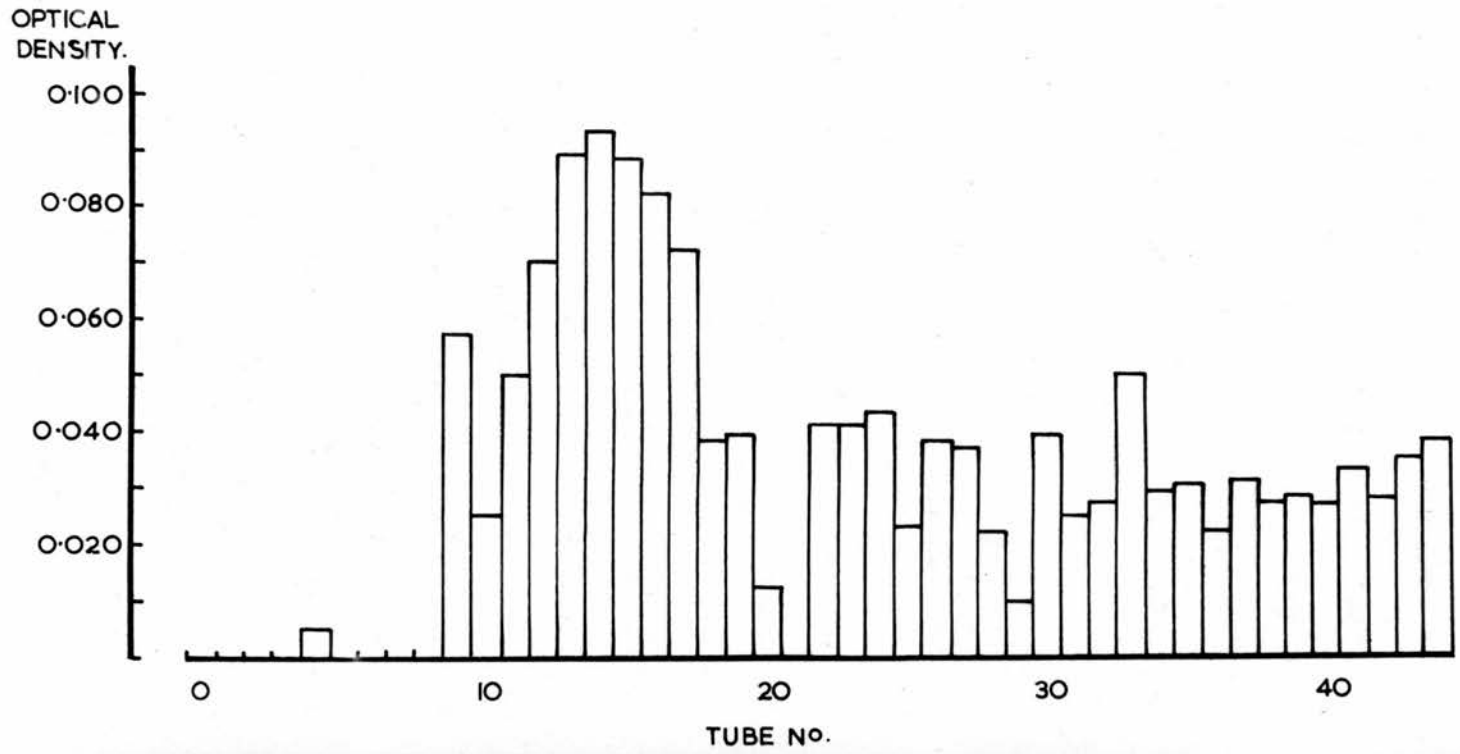
The individual fractions were pooled in the form indicated by Fig. 11 to give the combined fractions a, b, c, d and e, which were dissolved in 100 µl. of methanol each; 5, 15,30, 10 and 5 µl. of the corresponding solutions were applied to the starting line of chromatograms which were run in Bush's system B_2 in duplicate. One of the chromatograms was sprayed with methanolic soda with negative results. The other sheet, containing compounds S and A as standards showed/

COUNTER-CURRENT DISTRIBUTION OF A BOILED-URINE. FIG. II.

EXTRACT IN THE SYSTEM.

- METHANOL – 80
- WATER – 20
- BENZENE – 85
- CHLOROFORM – 15

a b c d e



showed two reducing zones in fraction a and none in the others. One of the two zones was very faint and very 'polar'. The other, rather more intense, was a streak beginning a little above the upper limit of compound S spot and ending at the middle of compound A spot. Unfortunately the paper was overrun and no R_F values could be calculated. Therefore the remainder of fraction a was dissolved in 0.4 ml. of methanol and a new chromatogram run in the same system with compounds B, S and A as standards, using 1/20 of the solution of fraction a. The R_F values for the standard were, respectively, 0.35, 0.38 and 0.53, but mixed compounds B and S were not resolved. No yellow fluorescent spot was detected in fraction a after Bush's test was applied. The BT spray revealed an intense band between compounds S and A (0.39 to 0.57) with a faint tail (0.21-0.39). Attempts to obtain crystals from fraction a were unsuccessful.

Second Countercurrent Distribution.

For this second experiment a pool of 300 l. of male urine was collected. Night urine was not included. Each day's collection, averaging 6 l., was/

was extracted three times with $CHCl_3$, one-third of its volume. Any emulsions formed were broken by centrifugation at 2000 r.p.m. for 5 min. The extracted urine was boiled for 30 min., cooled and extracted three times with one-third of its volume. The chloroform extracts obtained before and after boiling were combined separately and washed twice with 0.2 vol. of N/10 NaOH and twice with water, dried with anhydrous sodium sulphate, filtered and evaporated to dryness under reduced pressure on a water bath. The dry residues were transferred to test tubes and kept in the refrigerator.

At the end of the collection, all the individual fractions were pooled together, and the resultant crude extracts of unboiled and boiled urine were weighed. Each extract was then dissolved in 1000 ml. of ether, the ether solutions being washed twice with 125 ml. of N-HCl, twice with N-NaOH and twice with water and then evaporated to dryness and weighed. Two aliquots of each extract representing 1/400 of the total weight were taken for formaldehyde determinations. The numerical data corresponding to these operations/

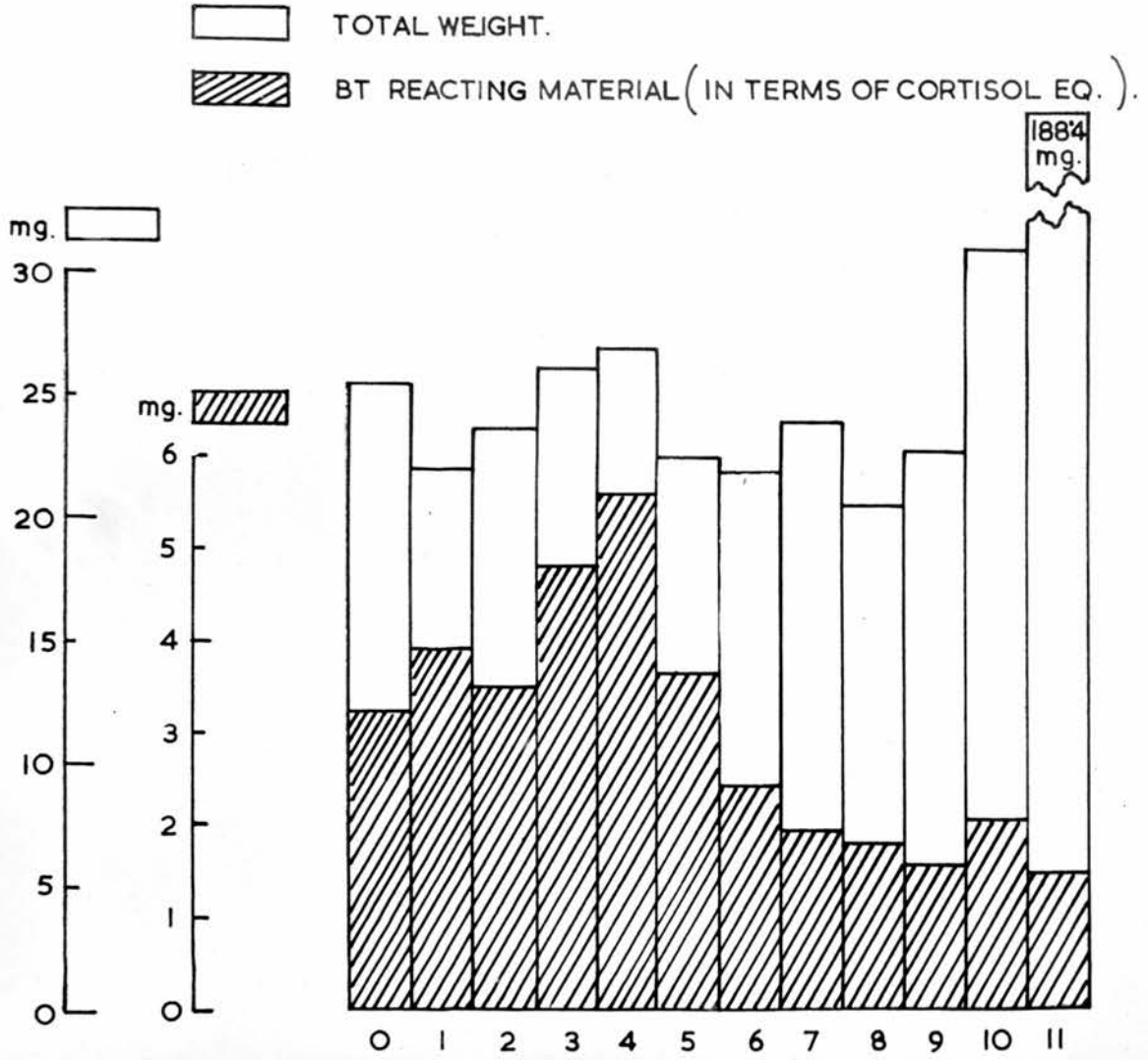
operations are included in Table 9 (p. 89).

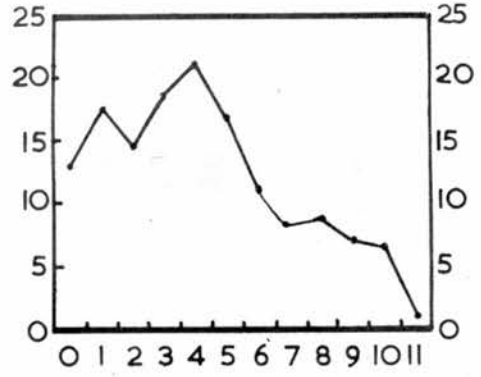
The countercurrent distribution was carried out in twelve 500 ml. separating funnels each containing 200 ml. of the upper (methanolic) phase of the system methanol, water, benzene and chloroform in the proportions (v/v) 80:20:85:15. The dry residue of the washed 'boiled urine' extract was introduced into the first funnel dissolved in 200 ml. of the lower phase of the above system. After vigorous shaking the two layers were allowed to separate, the lower layer transferred into the next funnel, and 200 ml. of fresh lower phase poured into the first funnel. Both funnels were shaken, the lower layers transferred one funnel further, and fresh lower layer added to the first funnel. In this manner, 11 transfers were carried out and 12 fractions of the 'boiled urine' extract were obtained by evaporating the contents of each funnel. An aliquot of 1/25 of each fraction was employed for the determination of the reducing material by the method of Chen et al. (1953). The results are given graphically in Fig. 12. The numerical data are included in Table 10 (p. 90). It is customary/

Table 9. Fractionation of the extract of 300 l. of urine for the second countercurrent distribution.

Fraction	mg.	mg./l.
UNBOILED URINE:		
Crude extract	1761.0	5.870
Washed extract	330.0	1.100
FSS (as compound S)	17.7	0.059
BOILED URINE:		
Crude extract	1357.0	4.523
Washed extract	492.0	1.640
FSS (as compound S)	30.4	0.101

FIG. 12.





LEFT: COUNTER-CURRENT DISTRIBUTION OF A BOILED-URINE EXTRACT IN THE SYSTEM:

METHANOL.	80
WATER.	20
BENZENE.	85
CHLOROFORM.	15

ABOVE: PERCENTAGE OF BT REACTING MATERIAL IN EACH FRACTION.

Table 10. Countercurrent distribution of the 'boiled urine' extract of 300 l. of male urine.
W = Weight in mg.
BT = Reducing material, as mg. of compound F.

Fraction No.	BT	W	BT.100/W
0	3.22	25.4	12.7
1	3.90	21.9	17.8
2	3.48	23.5	14.8
3	4.76	26.0	18.3
4	5.55	26.7	20.8
5	3.63	22.3	16.3
6	2.37	21.8	10.9
7	1.93	23.7	8.2
8	1.75	20.3	8.6
9	1.52	22.6	6.7
10	2.00	30.8	6.5
11	1.45	188.4	0.8
Total	35.56	453.4	7.8

customary and convenient to designate the first unit, when plotting a distribution curve, as 0.

Fractions 1-5 were combined together for further experiments to be described in the following section.

Discussion.

No similar data are available in the literature on the fractionation of 'boiled urine' extracts. Therefore, our discussion will be restricted to an evaluation of the results of both distribution experiments in themselves and compared with the results obtained by means of paper chromatography.

First of all, it will be seen that in both experiments the bulk of the reducing material containing the substance(s) which we have designated as X is concentrated at the left of the distributions, and not in the middle as expected from the partition ratios for compounds S and A. As Gregory and Craig (1951) have stated, the position of the peak of a pure substance in a distribution is much more accurate a measure of its partition ratio than any determination based on a single partition. Thus, it/

it may well be that the error in the determination of the partition ratios for compound S and A was considerable. On the other hand, the faulty calibration of the glass units may have had some effect in the displacement of the peak towards the left in the case of the first distribution.

The results of the first distribution are comparable to those obtained by means of paper chromatography because the way in which the urine extracts were obtained were very much the same. An inspection of Tables 5 and 8 reveals that the average of the formaldehyde determinations in the extracts submitted to paper chromatography agrees quite well with the corresponding values in the pool fractionated by countercurrent distribution. The ether residue of the 'unboiled urine' extract in this pool contained 0.195 mg./24 hr. of FSS, expressed as compound S, or 17.0 μg./24 hours of formaldehyde liberated after periodic acid oxidation. The corresponding average for the six urines in the paper chromatography studies was 19.6 μg./24 hours. Similarly, the 'boiled urine' extract in the pool had an amount of FSS equivalent to 0.270 mg. of compound S per 24 hours, or 23.5 μg./24 hours of formaldehyde, which is consistent with 18.7 μg./24 hours, the average found for the paper/

paper chromatography series.

The fact that the urine for the second distribution was not collected as 24 hour specimens, but only during the day, and the different treatment it received for the extraction and purification, preclude proper comparison with either the urine pool for the first distribution or the paper chromatography series. It can be seen from an inspection of Tables 8 and 9 that the amount of FSS in the 'boiled urine' extract (in mg./l.) of the second pool is about half that of the first. This is undoubtedly due to the larger diuresis during the day which outweighs the effect of the greater excretion of FSS during the daytime than at night.

The distribution curves permit the calculation of partition ratios from the position that the peaks occupy in them. It is also possible to calculate the amount of material giving a peak from its central value. In both cases the assumption is made that the peak or peaks correspond to pure substance(s). For practical purposes we may take the peak with a maximum in fraction no.14 in Fig. 11, and the peak with maximum in fraction no./

no. 4 in Fig. 12 as due to only a substance, X. As a matter of fact the examination by paper chromatography of these fractions did confirm that the major reducing material present in them was X, although other faint spots could be seen as well.

The partition ratio of X in the first distribution can be calculated by means of the formulae developed by Williamson and Craig (1947):

$$K = T_r/F.T_{r-1} \qquad (16)$$

$$K = F'.T_{r+1}/Tr \qquad (17)$$

in which K = partition ratio, T_r = the amount of substance in tube no. r of the distribution, T_{r-1} and T_{r+1} the values for the adjacent tubes to r, and $F = (n + 1 - r)/r$; (18)

$$F' = (r + 1)/(n - r) \qquad (19)$$

where n is the number of transfers of the distribution.

Applying these formulae to the data represented in Fig. 11, and taking r = 14, the two values of K for X agree within ± 0.005, and the average is 0.472.

We/

We can now substitute this value in the following equation, derived from that given by Gregory and Craig (1951):

$$Q = y_o \sqrt{2\pi nK/(K+1)^2} \qquad (20)$$

where Q is the total amount of the reducing material with a maximum in tube no. 14; y_o is the amount of reducing material in this tube; $n = 44$, and $K = 0.472$. The optical density for one-fifth of the contents of tube no. 14 was 0.093, equivalent to 33.2 μg. in terms of the standard used, as 25 μg. of compound F gave an O.D. = 0.070. The value for y_o in equation (20) will then be 166 μg., and Q = 1290 μg.

This value is about half the total amount of reducing material recovered from the distribution (see Table 8) and means a daily excretion of X equal to 64.5 μg., or 46.7 μg./l. This value is lower than that obtained by Fotherby (see Table 6), and than our own estimates from visual comparison of the size and intensity of the spots in previous chromatograms with those of known amount of standards. The explanation may lie on some degree of lability of the substances, losses during the manifold operations involved in the/

the distribution, and differences in reactivity with BT in the test tube and on paper. The theoretical curve fits the experimental data well enough to permit the elimination of any gross error in the distribution.

To the peak with a maximum in tube no.4 in the second distribution correspond K values of 0.583 and 0.466 when formulae (16) and (17) are applied. It should be remembered that a distribution curve for such a small number of transfers does not take the Gaussian form unless the partition ratio is very close to 1.00, and that tube no.3 contains material belonging to the maximum in tube no. 1 (see Fig. 12). Therefore 0.466 seems a better value for the partition ratio of X, and the agreement with 0.472 obtained in the first distribution is remarkable.

The amount of X can be calculated from formula (20) with a result of 21 mg. or 70 µg./l. which is consistent with the value obtained from the series of urines submitted to paper chromatography, with Fotherby's results, and not in bad agreement with 46.7 µg./l. obtained in the first distribution.

Summary

a) Two countercurrent distributions of 'boiled urine' extracts from two pools have been carried out in the system methanol, water, benzene and chloroform (80:20:85:15).

b) The first distribution, of twenty pooled 24 hr. specimens of male urine with an average of 270 µg. of FSS/24 hr., was accomplished in 44 transfers, and showed a peak in fraction no. 14.

c) The paper chromatography of this peak revealed the presence of X as the major reducing material in this fraction.

d) The second distribution of 300 l. of daytime male urine (averaging 101 µg. of FSS/l.) was accomplished in 11 transfers, and showed a peak in fraction no. 4.

e) Further experiments, to be described in the next section, revealed the presence of X in this peak, as the predominant reducing material in this fraction.

f) /

f) The partition ratio for X in the system of solvents used was calculated from the first distribution, and the value, 0.472, was in agreement with the calculated value from the second distribution, 0.466.

g) The amount of X in the first pool was calculated to be 64.5 µg./24 hr. or 46.7 µg./l. Similar calculations for the second pool gave 70 µg./l.

h) The results are discussed in comparison with those obtained by means of paper chromatography.

Properties/

Properties of the Reducing Material Present in 'Boiled Urine' Extracts

This third section is a description of the results of several tests carried out on the reducing material - usually after rough purification by paper chromatography - in the hope that they would provide valuable information regarding the structure of the compound(s) in question.

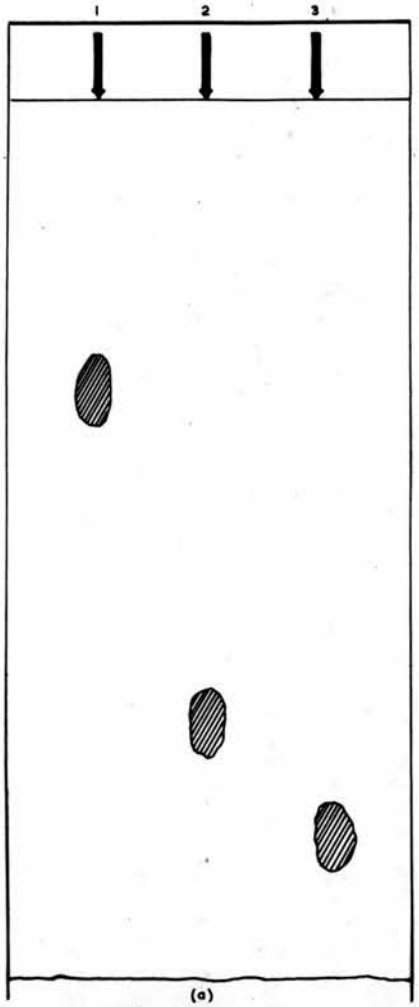
Chromatographic Properties

We have already made a distinction between X and Y in the preceding sections. Most of the properties to be described here refer to X, the material giving a strong reducing spot less 'polar' than compound S and more 'polar' than compound A. This material could not be separated into different spots when it was eluted from the paper and re-chromatographed in the same or other systems. A difficulty was found in that the substance did not show absorption when visualized in the ultraviolet lamp. Therefore, in this type of experiment, two duplicate chromatograms were always run, one of them being sprayed with BT to locate the position of the spots on the other in order to cut them out/

out for elution in cold methanol with occasional shaking for 18 hr.

Nine α-ketols have been studied in regard to their chromatographic properties to see how near they came to those of X. Compounds F, E, B, S, A and DOC have different R_F values, and all show a positive Bush's reaction with methanolic soda and absorption in the ultraviolet, characteristic tests for α,β-unsaturated ketones. The other three ketols studied are: pregnane-3α,21-diol-11,20-dione (TH-A), Δ^5-pregnene-3β,21-diol-20-one, and Δ^5-pregnene-3β,17α,21-triol-20-one. TH-A (Fig. 13b) is slightly more 'polar' than compound S in system B_2, their respective R_F values being 0.25 and 0.33, while the R_F of X is 0.48 in the same chromatogram. Fig. 13a is a copy of a chromatogram in the same system, where compound S and Δ^5-pregnene-3β,21-diol-20-one are shown to have, respectively, R_F values of 0.33 and 0.69. Finally, Δ^5-pregnene-3β,17α,21-triol-20-one has an R_F of 0.41 in the system benzene:70% aqueous methanol, that is, it is much more 'polar' than compound S (R_F = 0.69) and compound A (R_F = 0.76) in the same system, as

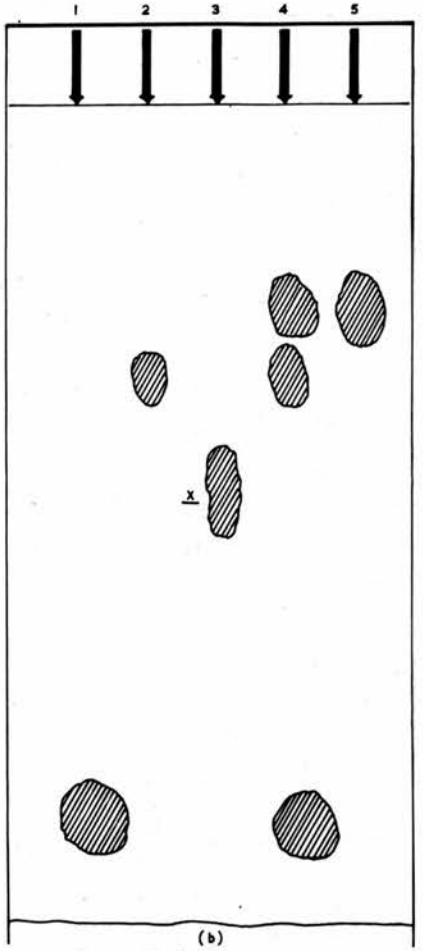
can/



(a)

BUSH'S SYSTEM B_2

1 = 10 µg. REICHSTEIN'S S.



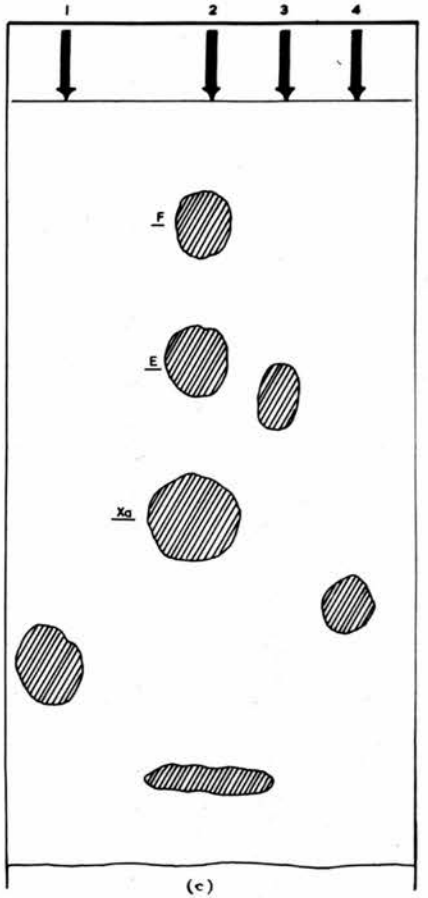
(b)

BUSH'S SYSTEM B_2.

1 = 10 µg. DOC.

2 = 5 µg. REICHSTEIN'S S.

3 = X (PEAK FROM COUNTER-CURRENT DISTRIBUTION).



(c)

BENZENE: 70% aq. METHANOL.

1 = 10 µg. KENDALL'S A.

2 = BOILED-URINE EXTRACT AFTER ACID TREATMENT. (without previous extraction of the urine.)

can be seen in Fig. 13c. None of these nine α-ketols is thus identifiable with X.

Ultraviolet Absorption Spectra

Two 24 hr. male urine specimens totalling 2.6 l. were extracted, boiled and extracted again as previously described. The crude 'boiled urine' extract was partitioned between hexane and 70% aqueous methanol, and the methanol-soluble fraction dissolved in 0.5 ml. of methanol and applied along the starting line of a chromatographic sheet (Whatman no.42) 13 cm. in width. After chromatography in the system benzene:70% aqueous methanol, two narrow strips were cut out from the sides to detect the BT positive bands. The band moving at a rate 0.49-0.59 that of the solvent front contained a faint spot, presumably Y. The material in the band moving at a rate 0.59-0.75 that of the solvent front was identified as X. The corresponding horizontal portions in the chromatogram were cut out and divided in small pieces which were left covered by methanol overnight.

The solutions were filtered and evaporated. The/

The residues were applied to separate papergrams 7 cm. in width as before and the papers run in the same solvent system. The detection of the bands was similarly carried out and the corresponding portions of the chromatograms cut out and eluted. In the chromatogram of Y this zone had a mobility relative to the solvent front of 0.48-0.63, the corresponding zone for X in the other chromatogram having a mobility of 0.57-0.72. From the chromatogram of X a portion of the paper near the starting line was cut out to be used as blank. The three eluates were evaporated to dryness, dissolved in 4 ml. of ethanol and their spectra read in a Unicam SP.500 as described in 'Methods'. The material eluted from the papers corresponds approximately to 70% of the amount present in the extracts.

The spectra showed a decrease in optical density from 220 mμ. towards longer wave lengths, with no maximum. Table 11 (p. 103) records the optical densities at some wave lengths.

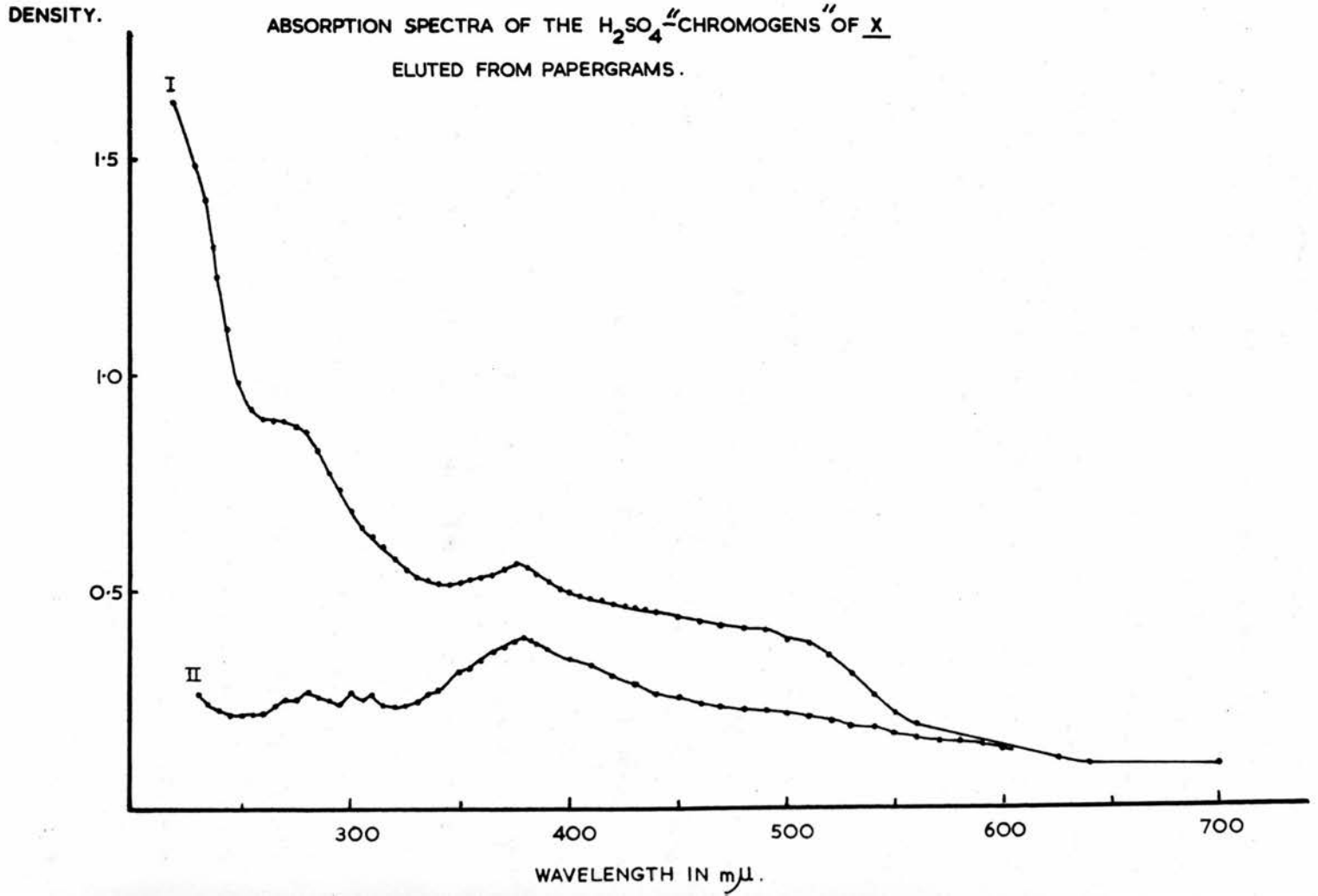
Another 24 hr. specimen of male urine was, as usual, extracted, boiled and extracted again. After hexane: 70% aqueous methanol partition, two/

Table 11. Ultraviolet absorption spectra of X and Y in ethanol. (The optical densities are corrected for the blank of the paper).

Wave length, mμ.	X	Y
225	1.135	0.535
245	0.683	0.503
255	0.507	0.442
265	0.469	0.307
280	0.425	0.270
300	0.262	0.098
350	0.067	0.011
400	0.028	0.001

FIG. 14.

ABSORPTION SPECTRA OF THE H_2SO_4 "CHROMOGENS" OF X ELUTED FROM PAPERGRAMS.



two-fifths of the methanol soluble fraction were applied to the starting line of a paper and chromatographed in system B_2 with 10 μg. of compound S as standard. The spot corresponding to compound S was located by examination of the chromatogram in the ultraviolet lamp, and X was detected by spraying a very narrow strip, 4 mm. in width, cut out from the margin of the chromatogram. The reducing spot had R_F = 0.39 (compound S, R_F = 0.32). The corresponding zone in the chromatogram, with a mobility relative to the solvent front of 0.30-0.48, was cut out. An equal portion of paper was taken from an uncontaminated region of the chromatogram to be used as blank. Both portions were eluted with 25 ml. of methanol overnight. The solutions were evaporated to dryness and transferred to test tubes to which 3 ml. portions of concentrated sulphuric acid (A.R., sp.gr. 1.84) were added. The solution of X was read against the blank in a Unicam S.500 spectrometer. Curve I of Fig. 14 was obtained. There was a maximum at 375 mμ., a minimum at 345 mμ., and inflections at 265-270 mμ., 470 mμ. and 540 mμ.

The/

The experiment was repeated with two-fifths of another 24 hr. male urine specimen, and the curve II of Fig. 14 is the graphic representation of the result. The fraction used for the readings was only one-fifth of the sample, the other one-fifth being used for the detection of the spot in the chromatogram. The sample of urine was collected entirely during the night. It can be seen that there is a maximum at 379 mμ., with some small, irregular peaks in the region 250-310 mμ.

Girard Separation

A 24 hr. sample of male urine, volume 1570 ml., was extracted, boiled and extracted again. The crude 'boiled urine' extract was submitted to the Girard separation according to the modification proposed by Pincus and Pearlman (1941), in which the formation of the hydrazones takes place at 90-100°C. for 20 min. in glacial acetic acid. The paper chromatograms of both the ketonic and the non-ketonic fractions failed to show any reducing spot at all. It was assumed that destruction occurred under these rather energetic conditions, and thereafter the method of Schneider (1950) was essentially/

essentially adhered to (see Fig. 4).

Another 'boiled urine' extract was treated in this manner, with the addition of a further extraction of the aqueous phase containing the steroid hydrazones 24 hr. after the acid for the hydrolysis was added. Neither this extract nor the non-ketonic fraction contained any reducing material judging from the paper chromatograms. The first ketonic fraction (obtained by extraction during the first two hours of acid hydrolysis of the aqueous phase) contained a reducing substance with an R_F value of 0.30 in system B_2 (R_F values for compound S and DOC in the same chromatogram were 0.34 and 0.88 respectively).

A third 'boiled urine' extract from a 24 hr. specimen belonging to the same subject was distributed between hexane and 70% aqueous methanol. The hexane phase was evaporated and transferred to a small test tube. The methanol phase was partially evaporated to remove as much methanol as possible, diluted with water, and extracted with ether. The aqueous phase was evaporated and transferred to a small test tube. The ether fraction was divided into two equal portions. A Girard separation was carried out on/

on one, the other being evaporated to dryness. Aliquots of each of these fractions - hexane, water, ether, and ketonic and non-ketonic fractions from the Girard separation - were applied to a chromatogram which was run in system B_2. No reducing material was observed in the hexane, water, and non-ketonic fractions. The ether fraction showed the spot corresponding to X with an R_F = 0.46, and the ketonic fraction a spot of similar intensity with an R_F = 0.27. Some reducing material remained near the starting line. The R_F values of the three standards used, TH-A, compound S and DOC were 0.22, 0.33, and 0.86 respectively.

Acid and Alkali Treatments

A 'boiled urine' extract, after hexane-70% aqueous methanol partition, was applied to the starting line of a chromatogram with 10 μg. of compound S at each side. After running in system B_2, a longitudinal strip was cut out from each side, and then sprayed with BT to locate the position of the band of X, which was cut out from the chromatogram, and divided into two equal portions to be eluted separately with 10 ml. of methanol/

methanol. After filtration, the eluates were evaporated to dryness. To one of them a few drops of methanol and 25 ml. of 0.1 N-H_2SO_4 were added, and the solution left standing at room temperature for one hour. Two extractions with 25 ml. of chloroform were then carried out, the chloroform extracts were combined and washed once with 10 ml. of 0.1 N-NaOH and twice with 10 ml. of distilled water, evaporated to dryness, and an aliquot of the residue applied to a chromatographic sheet. An identical aliquot of the other eluate (untreated by acid) was also applied as well as compounds S and DOC as standards. The paper was run in system B_2, and the spray with BT revealed the familiar large X spot in the untreated eluate with an R_F of 0.53. The eluate treated by acid showed a spot of similar intensity and size with an R_F = 0.30; no reducing material was seen where X would have been, if present. The R_F values for the standards were 0.37 (compound S) and 0.91 (DOC). These results are diagrammatically represented in Fig. 15b.

Fig. 15a shows the result of one of the Girard separations performed. Two papergrams,

(a) /

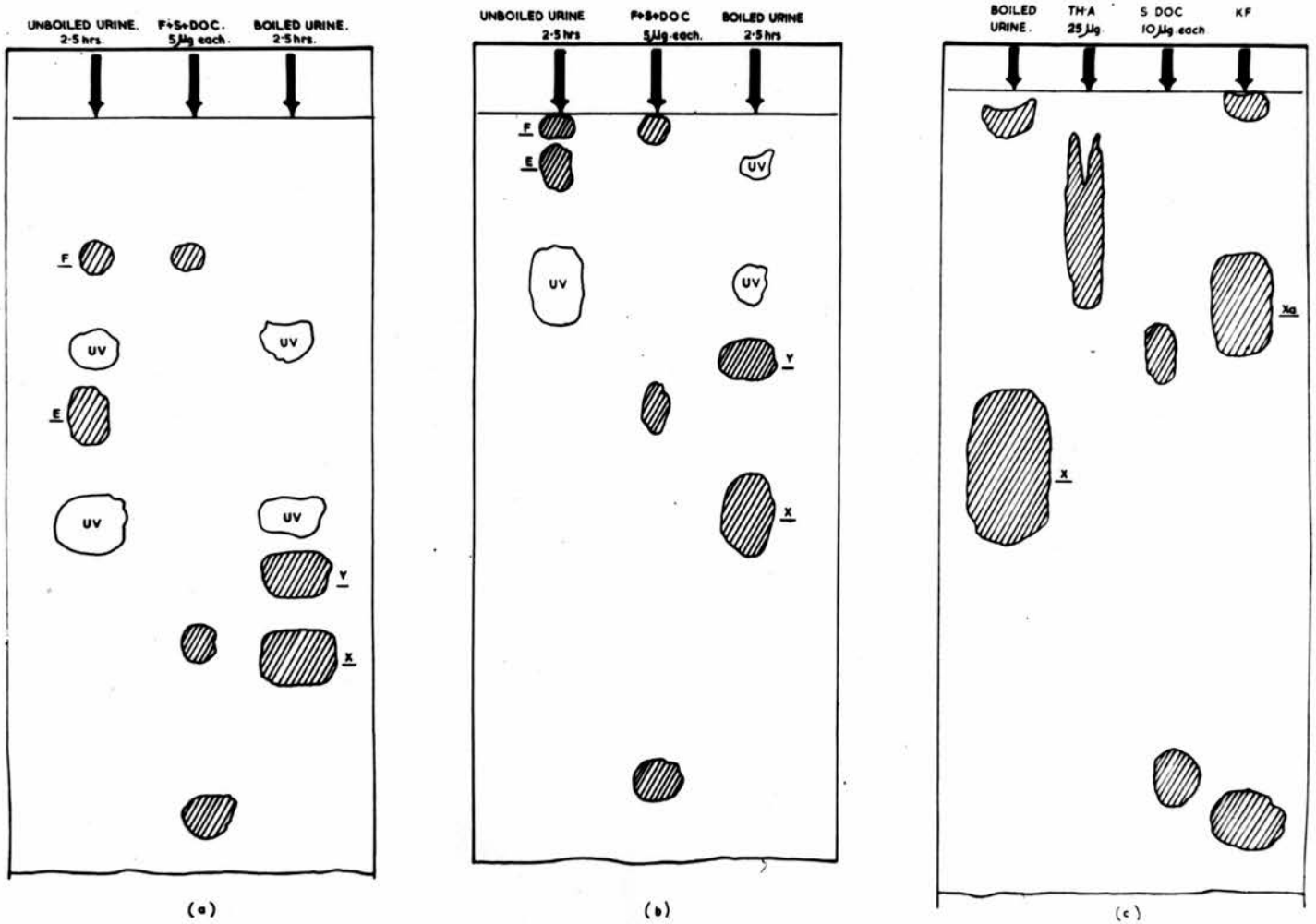
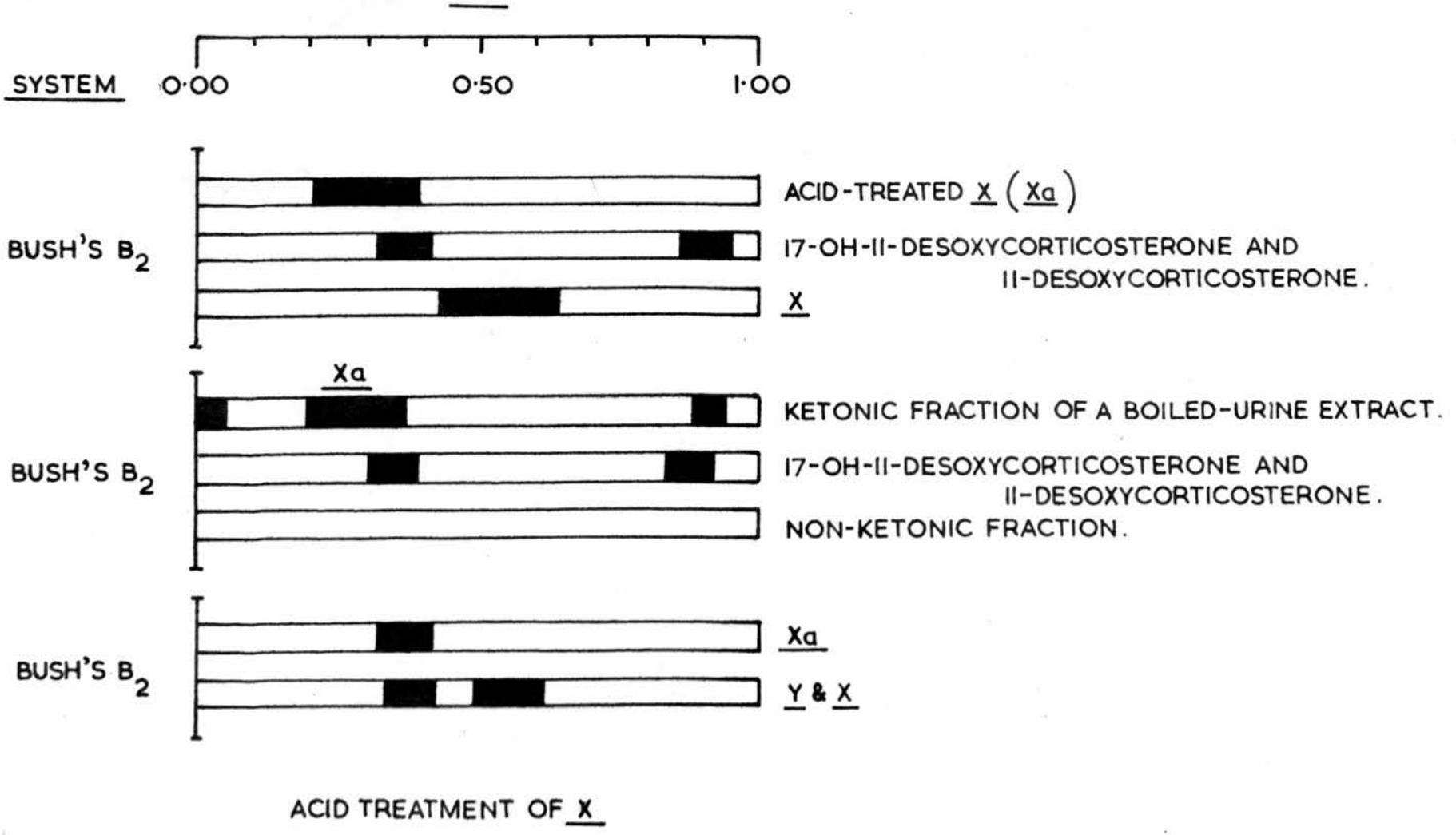


FIG 15a TYPICAL PAPERGRAMS OF NORMAL MALE'S URINE EXTRACTS (a) (b)


SYSTEM
0·00
0·50
1·00
BUSH'S B2
ACID-TREATED X (Xa)
17-OH-11-DESOXYCORTICOSTERONE AND
11-DESOXYCORTICOSTERONE.
X
Xa
BUSH'S B2
KETONIC FRACTION OF A BOILED-URINE EXTRACT.
17-OH-11-DESOXYCORTICOSTERONE AND
11-DESOXYCORTICOSTERONE.
NON-KETONIC FRACTION.
BUSH'S B2
Xa
Y & X
ACID TREATMENT OF X

(a) and (b), of untreated urine are included. There can be little doubt that the reducing spot seen in the ketonic fraction is identical with that obtained after acid treatment of X. This product will be called Xa. Its running rate is very similar to that of Y, as can be seen in the lower part of Fig. 15b, representing an experiment in which Y and X from a 'boiled urine' extract are compared with Xa obtained by acid treatment of a fraction of X of the same extract eluted from a previous chromatogram.

A 'boiled urine' extract was divided into four portions, and three of them were treated by 25 ml. of 0.1 N solutions of NaOH, $NaHCO_3$, and NA_2CO_3, after addition of a few drops of methanol to dissolve the dry residues. The solutions were neutralized after one hour with 2.5 ml. of N-HCl, extracted twice with 50 ml. of chloroform, and the combined chloroform extracts washed with water, dried and evaporated under reduced pressure. The chromatogram did not show any difference between these portions after the alkali treatment at room temperature and the untreated portion.

An experiment was designed to see if the conversion of X into Xa was reversible. Three-

fifths of an extract of boiled urine, corresponding to a 24 hr. urine sample, were acid-treated and the conversion product was divided in three equal portions which were applied to a paper, together with one-fifth of the untreated boiled urine extract. The chromatogram was cut along its length in two halves; one of them containing one of the spots after acid treatment and the untreated portion was sprayed with BT. Xa was eluted from the other half of the paper, one of the spots being treated by 0.1 N-NaOH as described in the preceding paragraph, and the other kept as reference. A final chromatogram was run with the untreated portion, the acid-treated portion, and the acid and alkali-treated portion, each one representing one-fifth of the 24 hr. 'boiled urine' extract. The alkali treatment did not modify the Xa spot.

Digitonin Precipitation

When the procedure outlined in Fig. 5 was used on a 24 hr. 'boiled urine' extract, the chromatogram (Fig. 16a) showed that X was in the α-fraction; no reducing material was seen in the β-fraction. In a repetition of this experiment a faint spot was observed in the β-fraction, presumably/

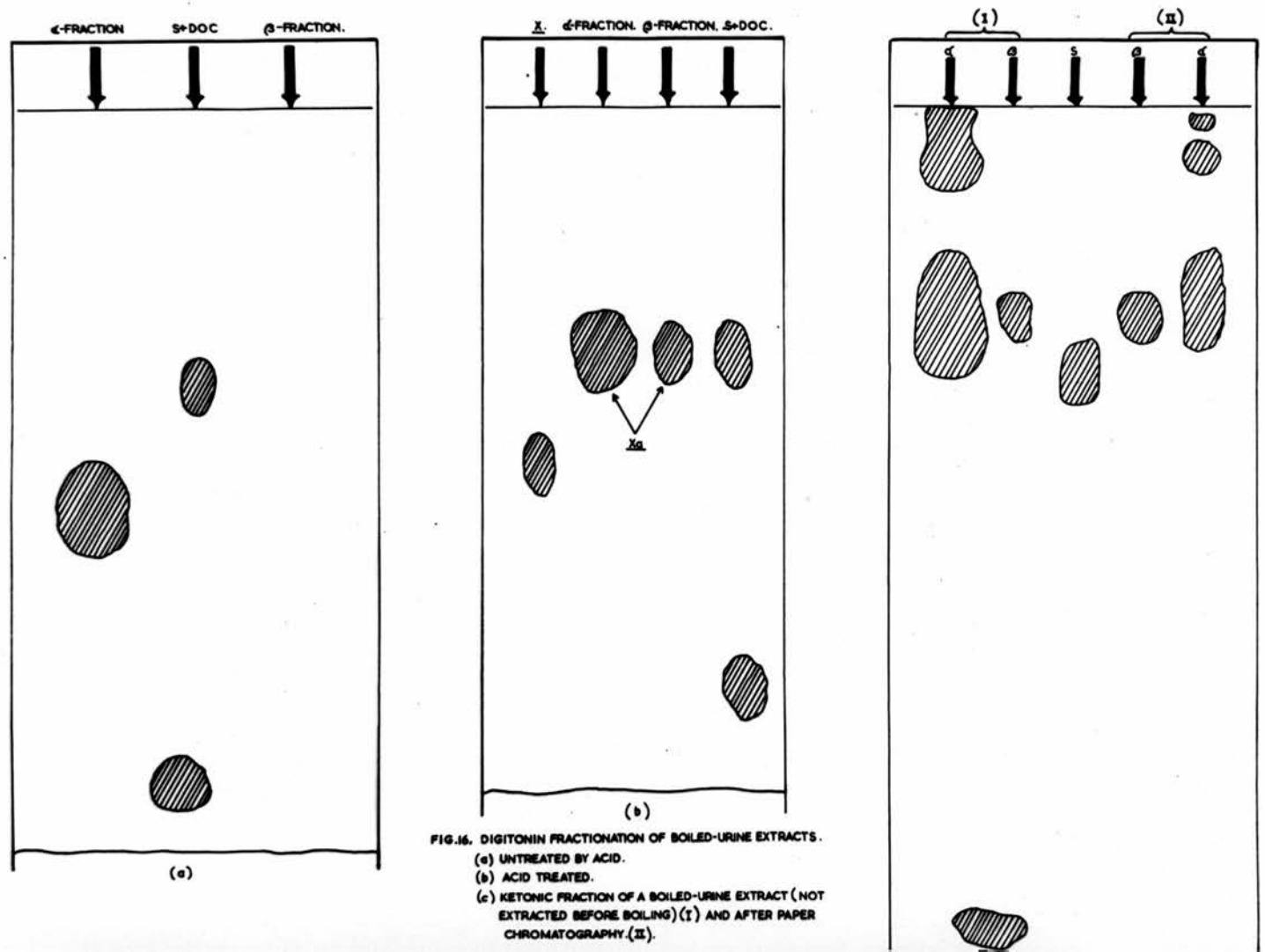
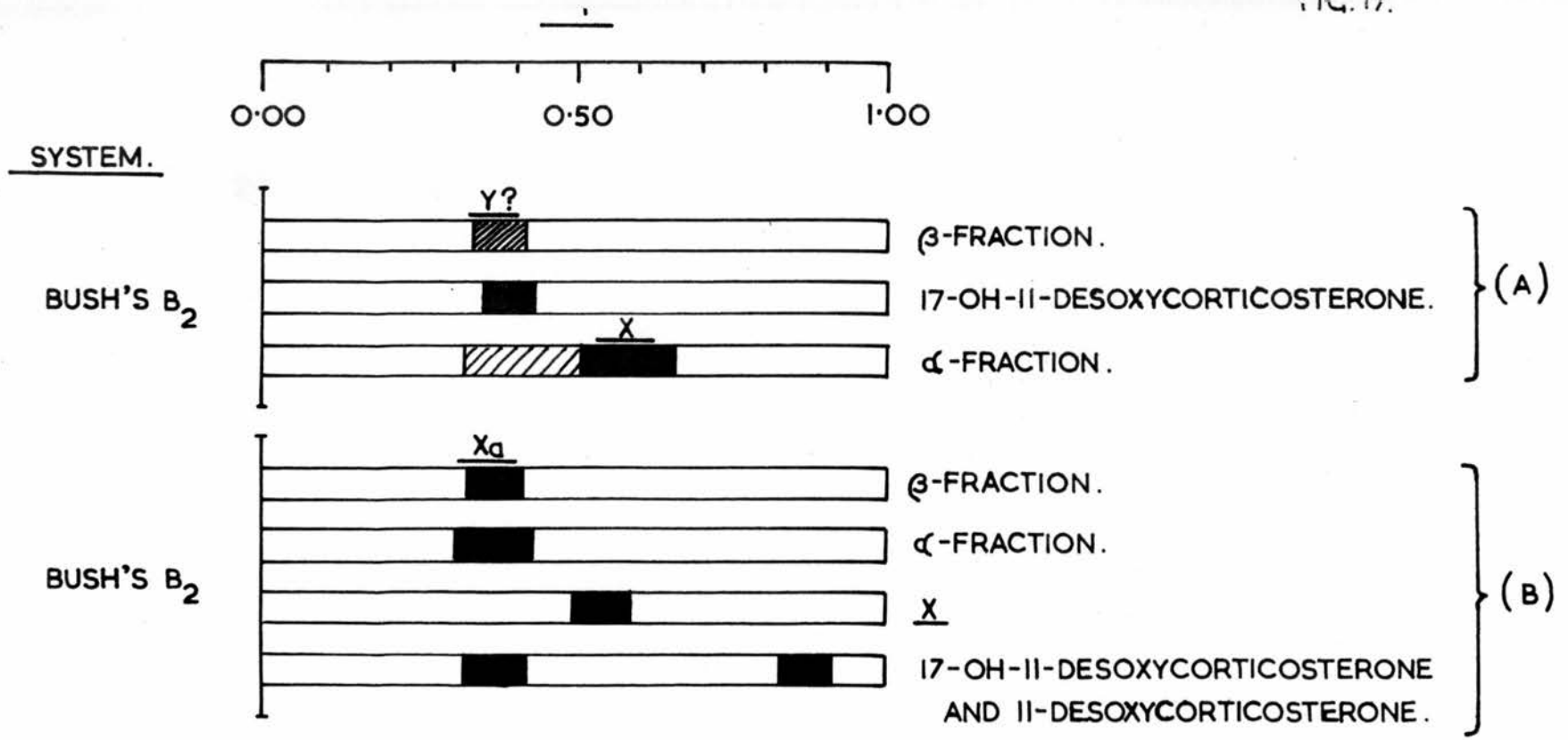


FIG.16. DIGITONIN FRACTIONATION OF BOILED-URINE EXTRACTS.
(a) UNTREATED BY ACID.
(b) ACID TREATED.
(c) KETONIC FRACTION OF A BOILED-URINE EXTRACT (NOT EXTRACTED BEFORE BOILING) (I) AND AFTER PAPER CHROMATOGRAPHY (II).



DIGITONIN PRECIPITATION OF:

A) BOILED-URINE EXTRACT.

B) BOILED-URINE EXTRACT AFTER ACID TREATMENT.

presumably due to Y; in the α-fraction, X was accompanied by a faint tail of a more 'polar' reducing material.

The digitonin precipitation of a 'boiled urine' extract after acid treatment at room temperature (Fig. 16b; Fig. 17B) revealed that a small portion of the material which we call Xa was in the β-fraction, the largest part remaining in the α-fraction. Fig. 16c is a copy of a chromatogram showing that previous purification of Xa did not appreciably modify the results.

Oxidation of Xa with Sodium Bismuthate

The oxidation of C_{21}-steroids by sodium bismuthate is a good test for the differentiation between 17,21-dihydroxy-20-ketocorticosteroids and those steroids lacking the hydroxyl group at C-17, for steroids of the former group yield 17-ketosteroids, while those of the second yield C_{20}-etioacids (Brooks and Norymberski, 1952). The oxidation with periodic acid would yield etio-acids whether a 17-hydroxyl group in addition to the primary α-ketolic side-chain existed or not, the reason being that, unlike sodium bismuthate and lead tetraacetate, periodic acid does not oxidize/

oxidize α-hydroxy acids. Sodium bismuthate has the advantage over lead tetraacetate that the oxidation can be carried out in an aqueous medium (Rigby, 1950).

A pool of 19.8 l. of daytime male urine was boiled and extracted. The extract was partitioned between hexane and 70% aqueous methanol, and a Girard separation was carried out on the methanol-soluble fraction. Papergrams with 1/100 (198 ml. of urine) of the resultant fractions proved that the ketonic fraction contained the usual Xa spot, while no reducing spot was seen in the non-ketonic fraction. Along the starting line of a chromatographic sheet, one-fifth of the ketonic fraction was applied in a band. After development in system B_2, the band corresponding to Xa was cut out, its position being found out by spraying two narrow strips from the sides of the chromatogram with BT.

Xa was then eluted from the cut portion with methanol, and the solution evaporated to dryness. One half of the dry residue was oxidized with sodium bismuthate in the way described by Brooks and Norymberski (1952). The reaction/

reaction mixture was extracted with chloroform, the extract evaporated, and two aliquots of the dry residue were applied to a chromatogram together with two equivalent aliquots of the untreated eluate, 10 μg. of compound S, and 10 μg. of dehydroepiandrosterone; compound S was at one side of the starting line and dehydroepiandrosterone at the opposite end, each half of the paper containing one spot of the untreated eluate and another spot of the oxidized portion. After development in system B_2, the paper was cut in two halves. The half containing compound S was sprayed with BT, and the other half with m-dinitrobenzene reagent (Axelrod, 1953b). The BT positive spot of Xa appeared in the untreated but not in the oxidized eluate. In the paper sprayed with m-dinitrobenzene the only purple spot was that of the standard dehydroepiandrosterone. The experiment was repeated with the same result. It appears that the group responsible for the reducing power of Xa is destroyed by sodium bismuthate oxidation without formation of a 17-ketosteroid.

Oppenauer/

Oppenauer Oxidation

As will be discussed later, there were reasons to think that Xa might be a 3β-hydroxy-Δ^5-unsaturated steroid. Oppenauer (1941) has described in detail the preparation of cholestenone from cholesterol. His method, reduced to adequate proportions for work at a micro-scale, was applied to the sodium bismuthate-oxidation product of Xa, hoping that the resulting Δ^4-3-ketosteroid would be easily identified by means of paper chromatography.

The method was first tested on dehydroepi-androsterone: 3 mg. of this compound, dried over P_2O_5 in a vacuum desiccator, were placed in a 25 ml. round-bottom flask with 7.5 ml. of dry acetone and 10 ml. of anhydrous benzene. The mixture was brought to boiling under reflux, and 800 mg. of aluminium t-butoxide dissolved in 5 ml. of benzene were poured down the condenser. The refluxing of the cloudy mixture was continued for 8 hours. The temperature was controlled by having the flask immersed in a bath of dibutylphthalate regulated at 80 ± 5°C. After cooling, 2 ml. of water and 5 ml. of 10% sulphuric acid were added; the contents were transferred to a 50 ml. separating/

separating funnel, 15 ml. of water were added, and the funnel was shaken thoroughly. The water layer was drawn off and extracted in another funnel with 10 ml. of benzene. The combined benzene layers were washed three times with 10 ml. of water, filtered through a layer of anhydrous sodium sulphate, and evaporated to dryness. The residue was dissolved in 3 ml. of methanol and 15 μl. of this solution applied to a chromatogram, with 15 μg. of dehydroepiandrosterone as standard. The oxidation product absorbed rather strongly when viewed at the ultraviolet light of 253 mμ. ('Chromatolite'). After running in system B_2 the ultraviolet inspection revealed two very faint spots in the middle of the paper, and a strong one near the solvent front. After spraying with m-dinitrobenzene two spots were detected, one with the same R_F as the standard dehydroepiandrosterone, and the other very strong and less 'polar' showing a perfect coincidence with the spot pencil-marked after ultraviolet inspection. This was considered satisfactory evidence for the partial conversion of dehydroepiandrosterone into Δ^4-androsten-3,17-dione. Two-fifths of the ketonic fraction described on

p. /

p.112 (= 7.92 l.) were submitted to paper chromatography in system B_2. The portion of paper containing Xa was cut out and eluted in the usual manner; the sodium bismuthate oxidation was carried out on the residue, and also on 1 mg. of 11-dehydrocorticosterone. The product of the sodium bismuthate oxidation of Xa was then oxidized by the method outlined in the preceding paragraph. An aliquot of the final product was chromatographed in system B_2 with the etioacid obtained by sodium bismuthate oxidation of compound A. The chromatogram failed to show any dark spot at the ultraviolet inspection. The experiment was repeated with another extract from 11.7 l. of daytime urine with an equally negative result.

Extraction of Urine at pH 1.0 and Room Temperature

This experiment was designed to confirm the hypothesis that Xa might be a 3β-hydroxy-Δ^5--steroid, obtained by the acid treatment of X, which in turn would be the corresponding 6β-hydroxy-3,5-cyclo compound. In other words, Xa would be to X what dehydroepiandrosterone is to 6β-hydroxy-3,5-cycloandrosten-17-one. It will be recalled (see Fig. 2) that continuous extraction of a solution/

solution of dehydroepiandrosterone sulphate at room temperature and pH 0.9 for 24-48 hr. yields 100% of pure dehydroepiandrosterone (Lieberman et al., 1954).

9.7 l. of male urine were adjusted to pH 1.0 with N-H_2SO_4, and continuously extracted for 5-6 hr. each of three consecutive days. The ether extract (3 l.) was washed twice with 0.2 vol. of N-NaOH, and three times with 0.2 vol. of water, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was partitioned between hexane and 70% methanol. The methanol soluble fraction was strongly pigmented (due to the acid hydrolysis employed) and its further purification before paper chromatography was necessary. Accordingly, a modification of the partition method outlined by Haines and Karnemaat (1954) was applied as follows: 60 g. of silica gel (chromatographic silica, 200/300 mesh) were mixed with 30 ml. of ethylene glycol with vigorous stirring. The mixture was suspended as a slurry in 200 ml. of cyclohexane saturated with ethylene glycol to pack a column of 34 x 2 cm. After thorough washing of the column with methylene dichloride and cyclohexane (both saturated with ethylene glycol), the methanol-soluble fraction of the urine extract was added onto the column dissolved

in/

in 5 ml. of methylene chloride. The discontinuous gradient elution described in 'Methods' was used. Portions of 25 ml. were taken from a flask initially containing 900 ml. of cyclohexane, and transferred to the column. For each withdrawal, 10 ml. of methylene dichloride were added to the flask and mixed thoroughly. The rate of flow from the column was 5 ml./min. and 59 fractions of 25 ml. each were collected. In a pilot experiment, compound S was eluted from a similar column in the fractions with a concentration of methylene dichloride in cyclohexane between 10 and 30% (v/v). As Xa is only slightly more 'polar' than compound S, fractions with this proportion of eluants should contain it. Therefore, the 59 fractions were combined in four groups, A, B, C and D. Group B corresponds to the aforesaid range of concentrations. The fractions of which each group consisted and their weights were as follows:

Group	Fraction no.	Weight, mg.
A	1-8	14.4
B	9-25	19.6
C	26-49	12.7
D	50-59	3.0

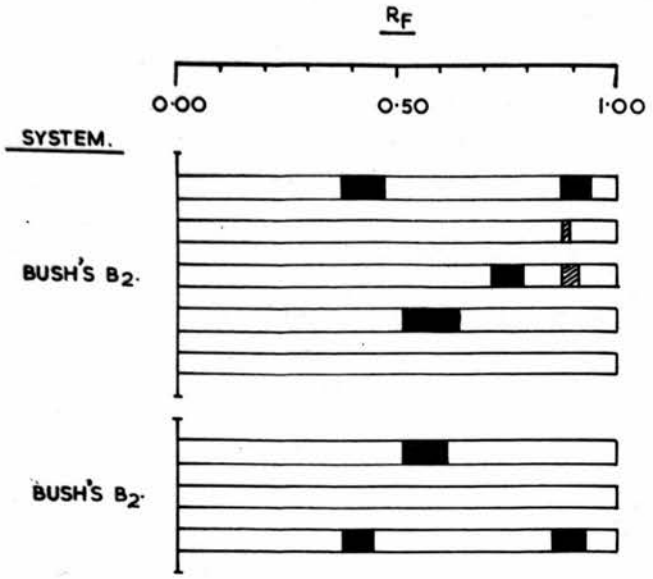
For the paper chromatography, 1/50 of

each/

each fraction (equivalent to 194 ml. of urine) was applied in duplicate to a paper with compound S and DOC (10 µg. each) as standards. The papers were run in system B_2. Probably because the system had been prepared about five weeks before this experiment was performed, the R_F values for compound S and DOC were 0.43 and 0.90 respectively, rather larger than usual. No BT-positive spots were observed in fractions A or D. Fig. 18 shows that fraction B contained reducing material with an R_F = 0.75, while fraction C contained a compound BT positive with an R_F = 0.58. The zones corresponding to this compound and to DOC in the duplicate papergram were cut out and eluted with methanol; a quantitative determination by means of the method of Chen et al. (1953) gave 0.407 mg. as the total amount of this substance present in fraction C. On this fraction, a Girard separation was done, and 1/40 of both the non-ketonic fraction (6.9 mg.) and the ketonic fraction (3.6 mg.) were taken to be chromatographed in system B_2 (Fig. 18). The reducing material was found in the non-ketonic fraction.

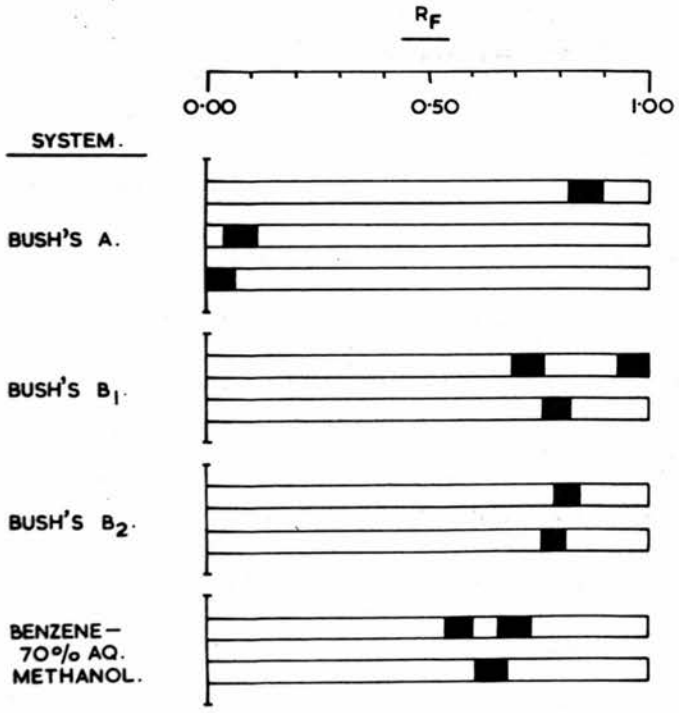
No spot with an R_F similar to that of Xa was/

FIG.18.



PARTITION CHROMATOGRAPHY OF URINE EXTRACT OBTAINED AT pH 1·0 & R.T. (CONTINUOUS EXTRACTION FOR 16 hrs.).
ANALYSIS OF THE FRACTIONS BY PAPER CHROMATOGRAPHY.

FIG.19.



PAPERGRAMS OF STEROID ACETATES.

was detected in any of the chromatograms, and the study of the fractions was not pursued further.

Discussion

The chromatographic properties of X are consistent with its having four oxygen atoms if, as it seems likely, it is a C_{21}-steroid. On this assumption, two oxygen atoms would be in the α-ketol side chain. The nature and the position of the other two oxygenated functions is a matter for speculation. By kind permission of Mr Fotherby (unpublished data) we include in Fig. 19 some results about the acetylation of X previously purified by paper chromatography. In some experiments, two spots were obtained on the papergrams of the acetylated X, one of them with an R_F similar to that of the monoacetate of compound S and the other much less 'polar' which might be a diacetate. In other experiments, only this last spot was observed.

These results may mean that X has an acetylable hydroxyl group other than that in C-21. Of course, the results can be explained admitting that X is a mixture of two reducing substances with the same R_F, one of them giving

a/

a more 'polar' acetate than the other.

The lack of a maximum at 240 mμ. in the ultraviolet absorption spectra of X and Y excludes the presence of an α,β-unsaturated ketonic group, always assuming that these reducing compounds are steroids. This result is in agrement with the absence of a positive reaction in Bush's test with the methanolic soda spray.

The ultraviolet absorption spectra of X in sulphuric acid showed a quite definite peak in the region between 375-379 mμ., apart from a high absorption beginning at 220 mμ. and decreasing rapidly towards longer wavelengths. An examination of the spectra recorded in the literature did not permit identification. It is noteworthy, however, that many of the 3β-hydroxy-Δ^5-unsaturated steroids listed by Bernstein and Lenhard (1953) have a maximum in the region between 350 and 410 mμ. In some compounds this peak is the highest and is very close to 375 mμ.; for instance: 377 mμ. for $\Delta^{5,7,9(11)}$-pregnatriene-3β-ol-20-one; 384 mμ. for $\Delta^{5,16}$-pregnadiene-3β-ol-20-one acetate. Δ^5-Pregnene-3β,21-diol-20-one diacetate has two peaks of similar height at 378 mμ. and 406 mμ. Dehydro-

epi/

epiandrosterone acetate has a high peak at 404 mμ.

While it would be unjustified to take these coincidences as evidence for the presence of a 3β-hydroxy-Δ^5-double bond in X, it is not unreasonable to suppose that if X had this grouping, a peak such as the observed one would very likely be present.

More significant are perhaps the results of the Girard separation. First of all, the hot treatment leads to a total destruction of the reducing properties of X, which cannot be found in either of the fractions resulting from the separation. It is well known that many corticosteroids, particularly those with a primary α-ketolic grouping, also show this lability. In the second place, the Girard separation carried out at room temperature yields a reducing ketone which is more 'polar' than X. This is the compound we have called Xa. As the reducing properties seem to be wholly preserved, the transformation must affect other parts of the molecule. An interesting possibility would be the transformation of an i-steroid into the 3β-hydroxy-Δ^5-unsaturated derivative. For instance, dehydroepiandrosterone is more 'polar' than i-androstanolone/

androstanolone (Savard, 1953). The treatment of i-androstanolone with hydrochloric acid at room temperature, however, yields mostly 3β-chloro-Δ^5-androsten-17-one (which is far less 'polar' than the parent compound), and less than 20% of dehydroepiandrosterone (Dingemanse and Huis in't Veld, 1952). The mechanism postulated by Lieberman et al. (1954) for the hydrolysis of steroid sulphates might explain the conversion of the i-compound into the 3β-hydroxy-Δ^5-unsaturated one if reversibility for the reaction between the intermediary radical and the i-compound is assumed. Thus, to recapitulate, if X is the i-compound, Xa cannot be the chloro-derivative, for this would be less 'polar' than the parent substance. Xa is more 'polar' than X, which is consistent with the hypothesis that Xa is the 3β-hydroxy derivative.

Whereas hydrochloric acid was usually employed for the hydrolysis of the Girard derivatives, sulphuric acid was used in some instances. The results, as far as the conversion of X into Xa is concerned, did not differ in those experiments in which sulphuric acid was used, lending further support to the hypothesis that/

that Xa is the 3β-hydroxy and not the 3-chloro derivative. The results obtained by treatment with 0.1 N-sulphuric acid lead to the same conclusion, always assuming that X is the i-compound.

Alkali treatment at room temperature for a short time did not appear to have any influence upon X. This kind of treatment also failed to reverse the conversion of X to Xa.

It is tempting to think that Y may be the same substance as Xa, because of the similarity in their running rates on the chromatograms. But we have not obtained any conclusive evidence to establish this identity.

In regard to the possible identity of Xa we should add that Fotherby (unpublished data) has found that Xa, purified by paper chromatography, gives a positive Pettenkoffer reaction in the modified version by Munson, Jones, McCall and Gallagher (1948), who claim that this colour test indicates the presence of unsaturation or potential unsaturation in ring B and a hydroxyl group or double bond in ring A.

The digitonin precipitation gave results which were not easy to interpret. It would appear/

appear that X is not precipitated in any case. The precipitation of Xa is rather incomplete and much more material appears in the α-fraction than in the β-fraction.[x] This in itself does not exclude that Xa is a 3β-hydroxysteroid, for many of them are recovered in the α-fraction, as we have already mentioned when describing the methods employed. It is unlikely that X is a mixture of two steroids, one with the 3-alpha and the other with the 3-beta configuration (which would explain the results), because in that case we have to assume that X is also a mixture of two reducing substances which under acid treatment would be transformed into another pair of compounds with the same R_F, or that X by acid treatment yields two different compounds with the same R_F.

The oxidation with sodium bismuthate seems to/

x However, using the same technique, Mr. Fotherby usually finds that Xa appears mainly in the β-fraction, only a small portion remaining in the α-fraction. The reason for this disconcerting difference is unknown.

to exclude the presence of 17-hydroxyl group, for in that instance a 17-ketosteroid would have been identified. The oxidation product of Xa was, however unidentified, because the etioacid which probably results from the sodium bismuthate treatment could not be detected even after Oppenauer oxidation which should convert the assumed 3β-hydroxy- Δ^5-unsaturated grouping into a Δ^4-3-ketone group. The reasons for the failure of the Oppenauer oxidation are not clear, but some alteration of the aluminium-t-butoxide used is not improbable, since other workers in the Department failed in obtaining satisfactory results with this reagent.

The expected result from the continuous extraction of urine at pH 1.0 and room temperature was the presence of Xa. The actual results of this experiment are in disagreement with this expectation. It is not impossible that during the three days of the experiment Xa was destroyed by the long exposure to acid dissolved in the ether layer. And, of course, it may be that Xa is not, as assumed, a 3β-hydroxy- Δ^5-unsaturated steroid. Nothing can be said about the non-ketonic reducing material found in this experiment, except/

except that in all probability, it is not of steroid nature at all.

Summary

a) From the chromatographic properties of X and those of its acetylated product, it seems ~~seems~~ likely that it has four oxygen atoms, two of them probably in the form of acylable hydroxyl groups.

b) Acid treatment at room temperature, e.g. the acid hydrolysis employed in a Girard separation, transform X into another more 'polar' reducing steroid, Xa. The reaction is not reversed by subsequent alkali treatment at room temperature. This conversion might be explained by analogy with similar well-known reactions assigning to X a 3,5-cyclo-6-hydroxy structure in rings A and B, while Xa would be the corresponding 3β-hydroxy-Δ^5-unsaturated derivative.

c) The ultraviolet absorption spectrum of partially purified X in concentrated sulphuric acid, the partial precipitation of Xa by digitonin/

digitonin, and a positive modified Pettenkofer reaction for partially purified Xa provide some evidence in favour of the structure mentioned in the last paragraph. The failure to obtain the α,β-unsaturated ketone after Oppenauer oxidation, and the absence of Xa among the reducing substances liberated by continuous extraction of urine at pH 1.0 and room temperature do not lend support to the hypothesis, but are not necessarily inconsistent with it.

d) The sodium bismuthate oxidation of Xa does not yield a 17-ketosteroid, which excludes the presence of a 17-hydroxyl group in the molecule. An α,β-unsaturated ketonic grouping can also be excluded (absence of a maximum at 240 mμ. in the ultraviolet absorption spectrum.

The/

The Isolation and Identification of Crystalline Compounds From 'Boiled Urine' Extracts

All the results described so far point to the existence in urine of some reducing material extractable by chloroform and bound in some combined form which is split by boiling the urine for 30 min. before the extraction. The presumptive evidence for this reducing material being of steroid nature is rather strong, but the conclusive evidence would obviously be the isolation of the substance(s) in question, and the identification according to the accepted criteria in the field of steroid chemistry.

With this goal in mind, several extracts of 'boiled urine were fractionated by adsorption chromatography, and some of the crystalline fractions obtained were submitted to the usual identification procedures. The results of these experiments are described in this section.

Chromatographic/

Chromatographic Procedures

The type of gradient elution used has already been described in a previous section. A mixture of magnesium silicate and Celite (1:1 by weight) was used as the adsorbent. The magnesium silicate (B.D.H.,for chromatographic purposes) was moderately activated by overnight heating at 120°C. Preliminary experiments using Reichstein's compound S as standard, showed that strong activation of the magnesium silicate (heating at 400° C. for 2 days) led to incomplete recoveries. The Celite was kept in an oven at 110°C. For the preparation of the columns the mixture of magnesium slicate and Celite was admixed with enough petroleum ether or benzene - whichever was to be used as the less polar solvent to begin the elution - to form a not too thick slurry which was poured into the column, already half-filled with the same solvent. After stirring the contents vigorously with a glass rod, the excess of solvent was drained off while the adsorbent mixture sedimented, the top of the column not being allowed to dry. The extract, dissolved in the minimum amount of the same solvent, was then added, or by what proved to be

a/

a better technique, adsorbed on a small amount of magnesium silicate-Celite mixture suspended as a slurry in a small volume of the solvent. The elution began with pure petroleum ether (B.P. 80-100°C.) or benzene, and ethanol was added in a pregressively increasing concentration, as the more polar solvent. At the end, two further fractions were usually collected by washing down the column with pure ethanol. A pressure between 80-140 mm. Hg was applied throughout the fractionation to obtain a rate of flow of 2-3 ml./min.

The fractions were evaporated under a jet of air. Aliquots of the fractions were run on paper-grams which were sprayed with BT reagent. The appropriate fractions were then combined and chromatographed again on smaller columns. The crystalline products were separated and re-crystallized until a satisfactory melting point was obtained.

Two pools of urine were fractionated following the preceding plan. The urine was boiled, without previous extraction, and extracted as described in 'Methods'. Usually, the crude extracts/

extracts of each day's collection, 6 l. on the average, were partitioned between hexane and 70% methanol, and the methanol-soluble fractions were kept dried in the deep-freeze.

Fractionation of the First Pool.

An extract of urine corresponding to 103.5 l. and weighing 1.0256 g. was distributed between hexane and 70% methanol, and the methanol-soluble fraction amounting to 444 mg. was fractionated into ketonic and non-ketonic fractions, using 2 g. of Girard's reagent T, and the procedure described elsewhere (see Fig. 4). Hydrochloric acid was the agent employed for the hydrolysis of the ketone hydrazones. The weights of the fractions were:

Non-ketonic = 199.6 mg.
Ketonic = 182.5 mg.

Crystalline material was obtained from a methanolic solution of the ketonic fraction. The identification of this ketone, K_1, will be described later.

Evaporation of the mother liquors left a residue, 121.2 mg. in weight. A magnesium silicate-Celite column, dimensions 13 x 1 cm., was prepared with 5 g. of the adsorbent mixture. The residue was/

was added onto the column dissolved in approximately 10 ml. of warm benzene. From a flask containing benzene 10 ml. portions were withdrawn and transferred to the column, and at the same time 2 ml. of an ethanol-benzene mixture (1:3, by volume) were introduced into the flask. In this manner, the concentration of ethanol in the eluting mixture increased continuously, slowly at first and progressively faster as the volume in the flask, initially containing pure benzene, decreased; 27 fractions of 20 ml. each were collected including two collected when the column was eluted with ethanol-benzene (1:3), and another three with pure ethanol. The analysis of the fractions by means of paper chromatography is represented in Fig. 20. Fractions 1-6 were combined and rechromatographed under identical conditions except that petroleum ether was substituted for benzene. The results are given graphically in Fig. 21. Fractions 7-11 of this second chromatogram were combined and transferred to a small conical flask. From a methanolic solution of the 12.3 mg. of material thus separated, 4.3 mg. of impure crystals melting at 149-168° were obtained. The crystals were/

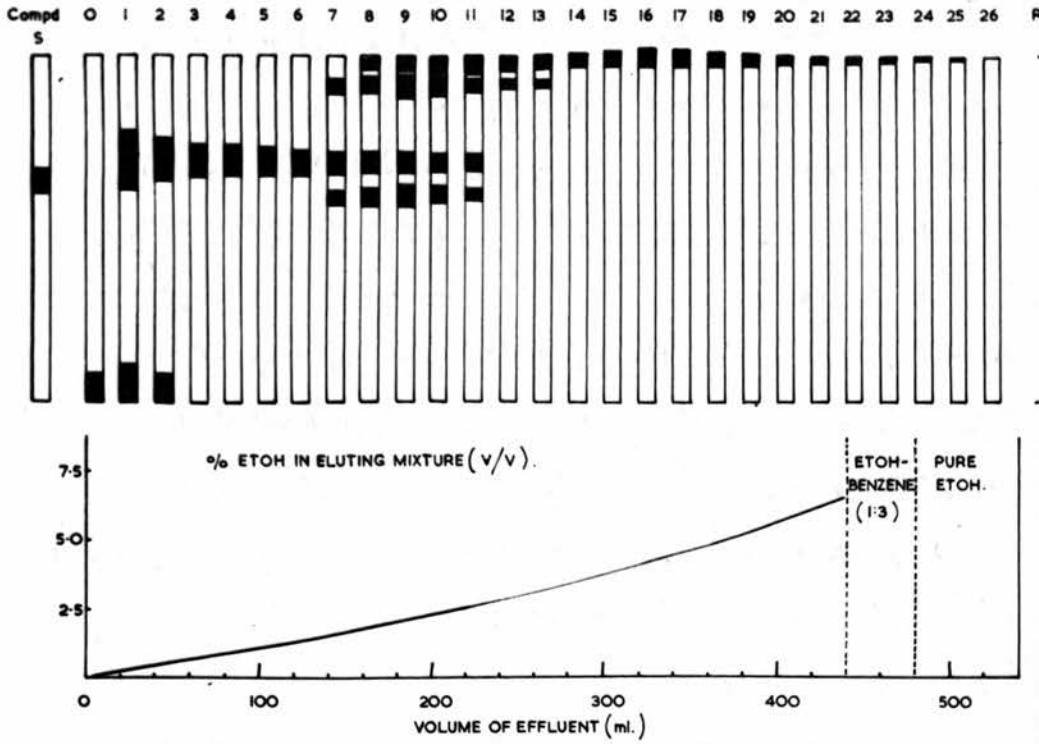


FIG.20. KETONIC FRACTION OF A BOILED-URINE EXTRACT CHROMATOGRAPHED ON A MAGNESIUM SILICATE-CELITE (1:1) COLUMN. PAPERGRAMS OF 1/25 OF THE FRACTIONS IN BUSH'S SYSTEM B_2.

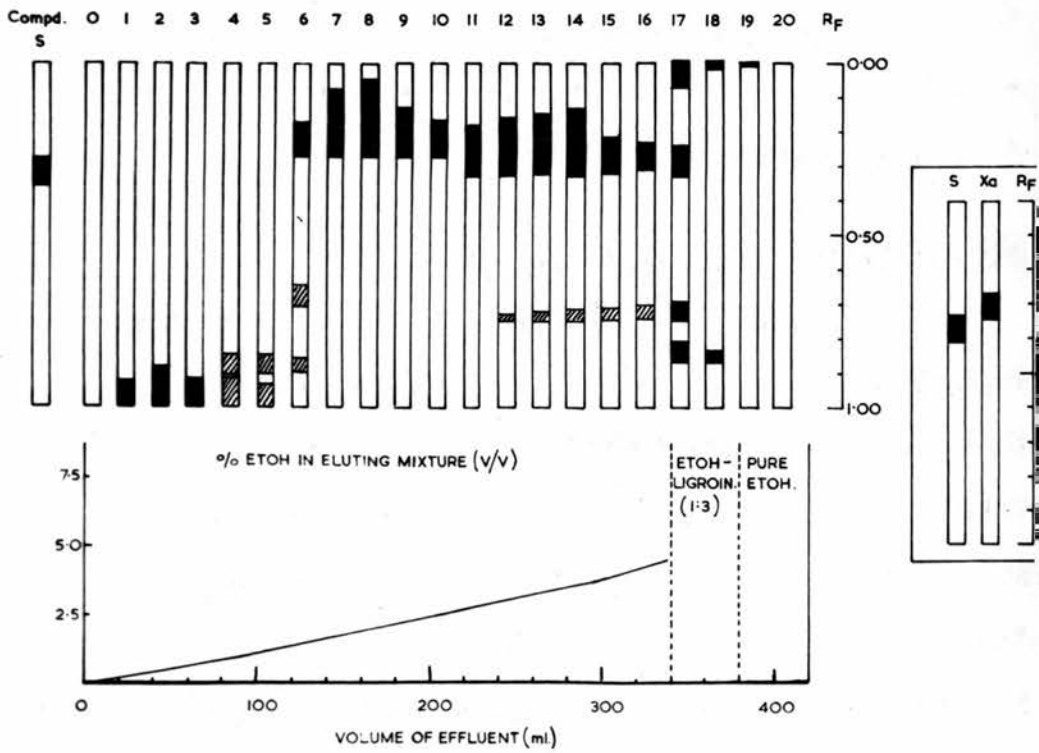


FIG 21 FRACTIONS 1-6 (FIG 20) RECHROMATOGRAPHED ON A MAGNESIUM SILICATE-CELITE (1:1) COLUMN. PAPERGRAMS OF 1/10 OF THE FRACTIONS IN BUSH'S SYSTEM B_2.

RIGHT: PAPERGRAM OF CRYSTALS FROM FRACTIONS 7-11 OF THIS SECOND CHROMATOGRAM.

were dissolved in 1 ml. of methanol and 5 μl. applied to the starting line of a chromatographic sheet. The result of this chromatogram is also represented in Fig. 21. It is worth noting that the time for the blue colour to appear after the spray with the BT reagent was longer for the crystalline material than for Reichstein's compound S used as standard. The intensity and size of the two spots were very much the same, in spite of the amount of crystalline material being twice that of compound S.

A second crystallization of this material - hereinafter called K_2 - from methanol yielded 3 mg. of crystals melting at 168-177°C. The experimental evidence for its identity will be described later.

Fractionation of the Second Pool

An extract of 118 l. of boiled urine was prepared in the usual manner, and a partition between hexane and 70% methanol carried out. The total methanol-soluble residue, weighing 573 mg. was submitted to a Girard separation as already described. The weight of the non-ketonic fraction was 261 mg. and that of the ketonic fraction 177 mg. No crystals could be obtained from a methanolic solution/

solution of the latter. Therefore, no K_1 was isolated in this second experiment.

The ketonic fraction was then chromatographed on a column about 20 x 2.5 cm. containing 50 g. of a mixture of magnesium silicate and Celite (equal parts, by weight). The adsorbent was poured onto the column as a slurry in petroleum ether (boiling range 80-100°C.). The elution was in the form of the 'discontinuous' gradient procedure described in the preceding paragraph with the exception that the volume in the reservoir was 1000 ml. instead of 500 ml. The eluate was collected in fractions of 50 ml. At the end, two fractions were collected eluting with ethanol-petroleum ether (1:3; v/v), and another two with pure ethanol. The residues from the fractions having an odd number were dissolved in 1 ml. of methanol each, and 1/100 aliquots of these fractions were chromatographed on paper (see Fig. 22). Only fractions 17 and 19 contained important amounts of blue tetrazolium-reducing material. The contents of tubes 16, 17 and 18 were combined and left aside. Fractions 11-15 and fraction 19 were combined and rechromatographed on a small column containing 5 g. of magnesium silicate/

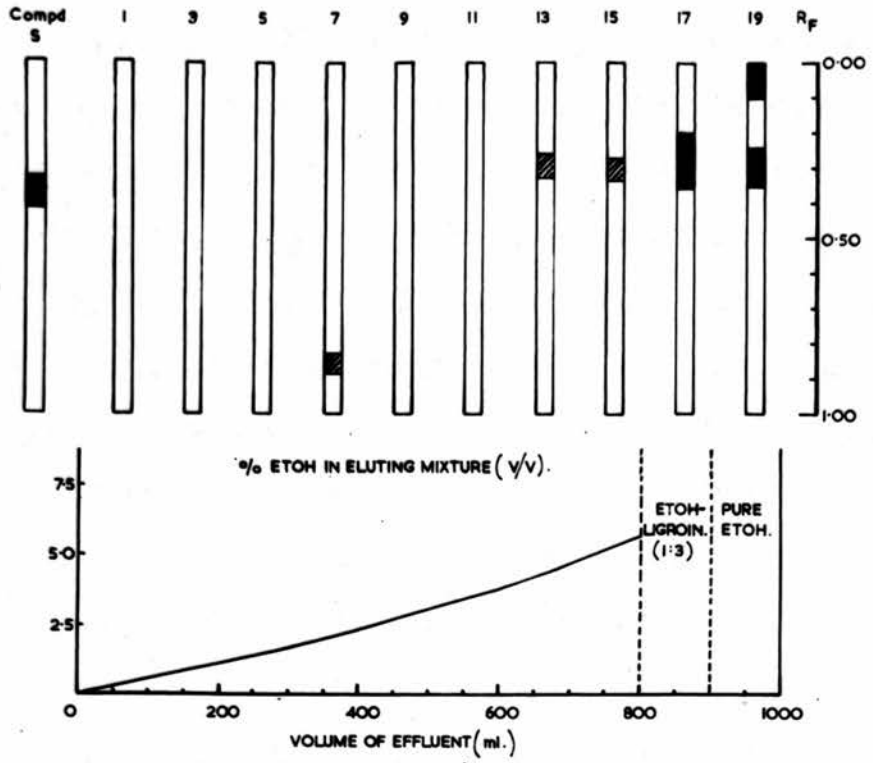


FIG.22. KETONIC FRACTION OF A BOILED-URINE EXTRACT CHROMATOGRAPHED ON A MAGNESIUM SILICATE-CELITE (1:1) COLUMN. PAPERGRAMS OF 1/100 OF THE FRACTIONS IN BUSH'S SYSTEM B_2.

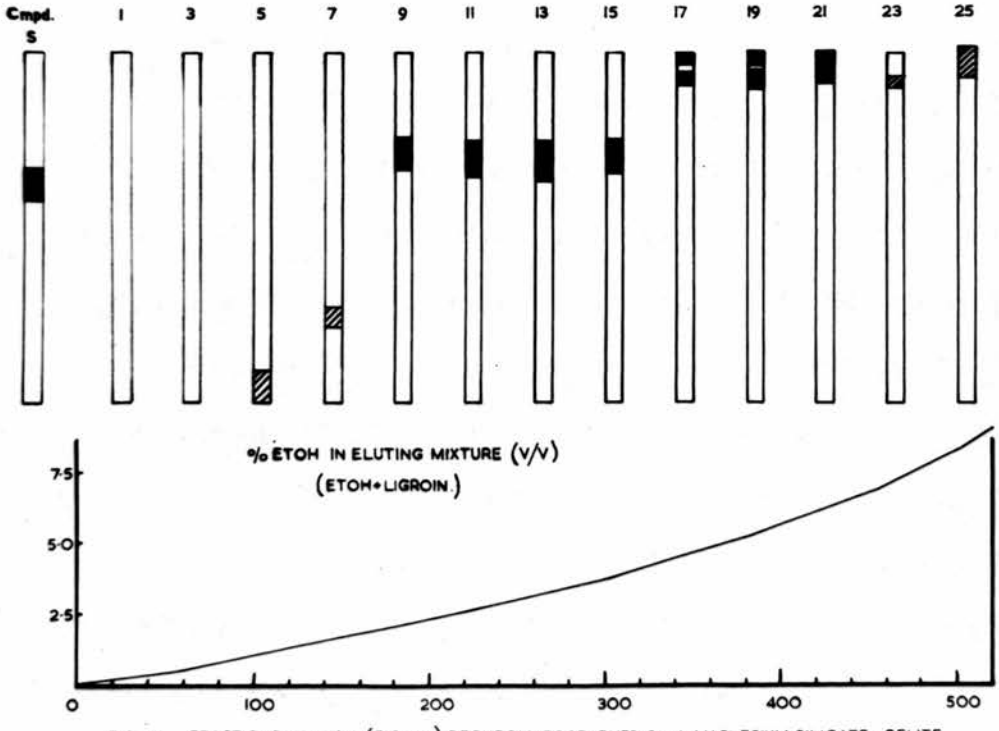


FIG 23. FRACTIONS 11-15 & 19 (FIG 22.) RECHROMATOGRAPHED ON A MAGNESIUM SILICATE-CELITE (1:1) COLUMN. PAPERGRAMS OF 1/25 OF THE FRACTIONS.

silicate-Celite mixture as adsorbent. The eluting solvents were petroleum ether and ethanol as before; 500 ml. of petroleum ether were placed in the mixing flask. The volume of the fractions was 20 ml. Alternate fractions were analysed for blue tetrazolium-reducing material by means of paper chromatography. The papergrams of 1/50 aliquots of these fractions are shown in Fig. 23. Fractions 8-16 of the second adsorption chromatogram were combined with fractions 16, 17 and 18 of the first (from which several attempts to obtain crystalline material failed), and the residue was chromatographed on another column with 5 g. of the usual adsorbent mixture. The elution was carried out with 80 ml. of benzene; 120 ml. of 1% ethanol in benzene; 40 ml. of 3% ethanol in benzene; 20 ml. of 25% ethanol in benzene, and 20 ml. of ethanol. No appreciable amounts of Xa were detected in the papergrams prepared with aliquots from these fractions. The material eluted by 1% ethanol in benzene was not examined by paper chromatography. Instead, these fractions were combined and crystals obtained from a methanolic solution of the residue. The crystals, about 3 mg., were similar to those of K_2 obtained

from/

from the first pool and melted, after a second crystallization, at 173-178°C. This material was then combined with K_2 and crystallized again from methanol. Approximately 4.5 mg. of a substance melting at 177-181°C. were obtained.

Identification of K_1 as 3β-Cl- Δ^5-androsten-17-one

This substance, obtained from the ketonic fraction of the first extract before it was submitted to chromatography, had a melting point of 147-150°C. This, as all the other melting points recorded here, is the uncorrected value obtained by means of a hot-stage melting point apparatus, usually giving results reproducible within ± 2°C. (Klyne and Rankeillor, 1947). The Zimmermann reaction (Callow, Callow and Emmens, 1938) showed that K_1 is a 17-ketosteroid (Fig. 24). The optical density at 520 mμ. of the colour obtained with 100 μg. of K_1 was 0.335, while an equal amount of dehydroepiandrosterone showed a value of 0.332. The two curves were identical.

The absorption spectrum of a solution of 120 μg. of K_1 in 4 ml. of concentrated sulphuric acid (A.R., sp. gr. 1.84), after 2 hr. at 25°C., is reproduced in Fig. 25. The shape of the curve/

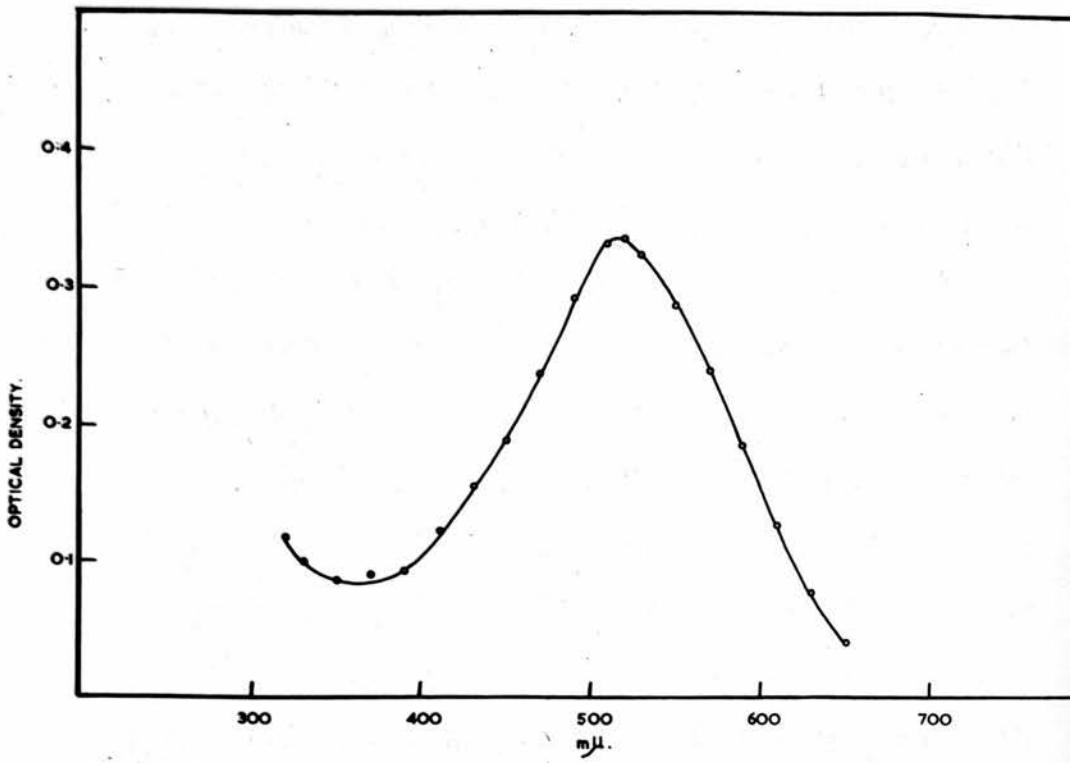


FIG.24. ABSORPTION SPECTRUM FOR THE ZIMMERMAN REACTION ON K_I.

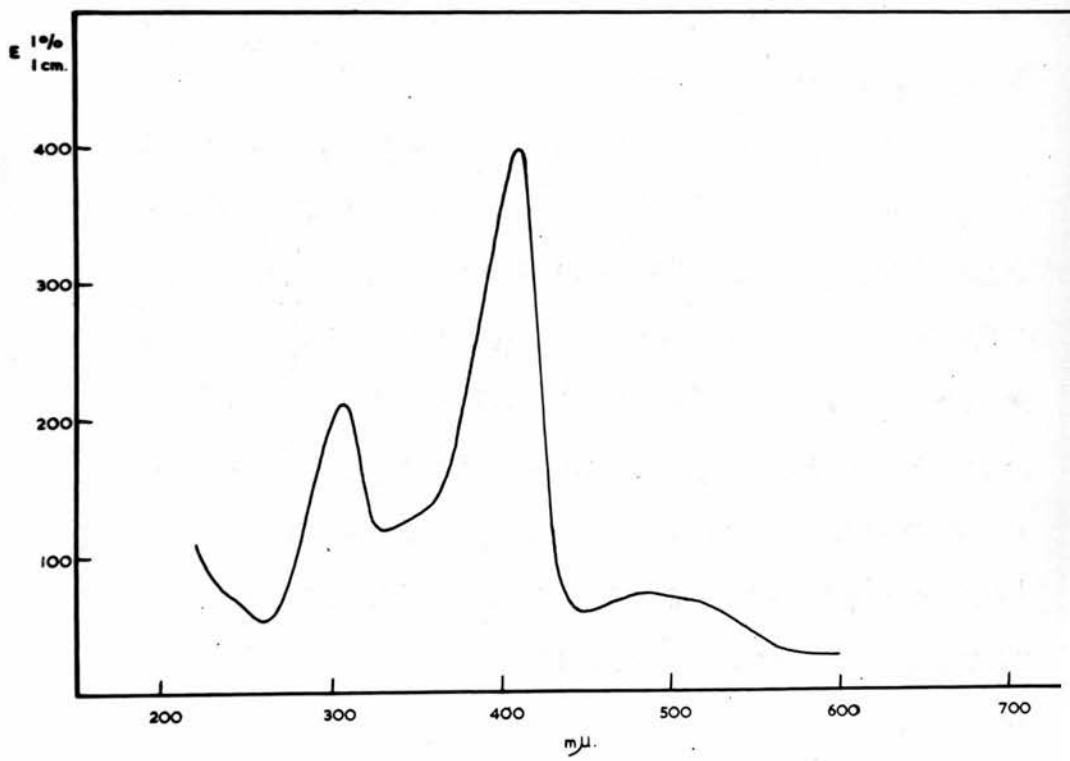


FIG.25. ABSORPTION SPECTRUM OF 120 μg. OF K_I IN 4ml OF CONC. H_2SO_4.

curve, and the position and relative intensities of the peaks correspond with those reported for dehydroepiandrosterone by Axelrod (1953b) and Bernstein and Lenhard (1953), although the extinction coefficients are smaller.

However, K_1 depressed the melting point of dehydroepiandrosterone. A careful study of the melting behaviour revealed that K_1 appeared to adopt a rod-like crystalline form at 125°C., tiny droplets were visible at 135°C., and the melting point was 145-152°C. Pure dehydroepiandrosterone (three times crystallized from methanol) showed no similar change in crystalline form; droplets were visible at 140°C., and the melting point was 147-149°C. A mixture of the two substances started to melt at 121°C.

The only known 17-ketosteroid with a melting point similar to that of K_1, and which might be expected to show the same absorption spectrum in sulphuric acid as dehydroepiandrosterone, is its 3β-chloro derivative having a slightly higher melting point. Accordingly, this substance was prepared by treatment of dehydroepiandrosterone with phosphorous pentachloride (Wallis and Fernholz, 1937). The melting point of the product/

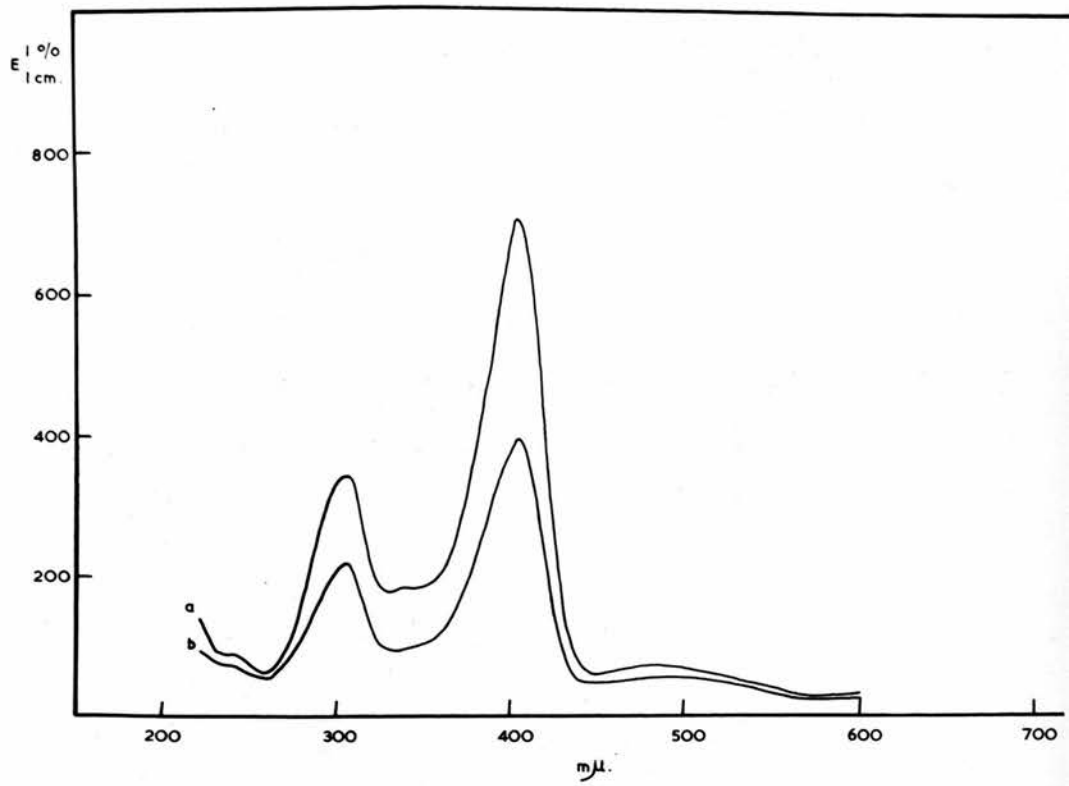


FIG.26. ABSORPTION SPECTRUM IN CONC. H_2SO_4 OF a) DEHYDROEPIANDROSTERONE.
b) 3β-CHLORO-Δ^5-ANDROSTEN-17-ONE.

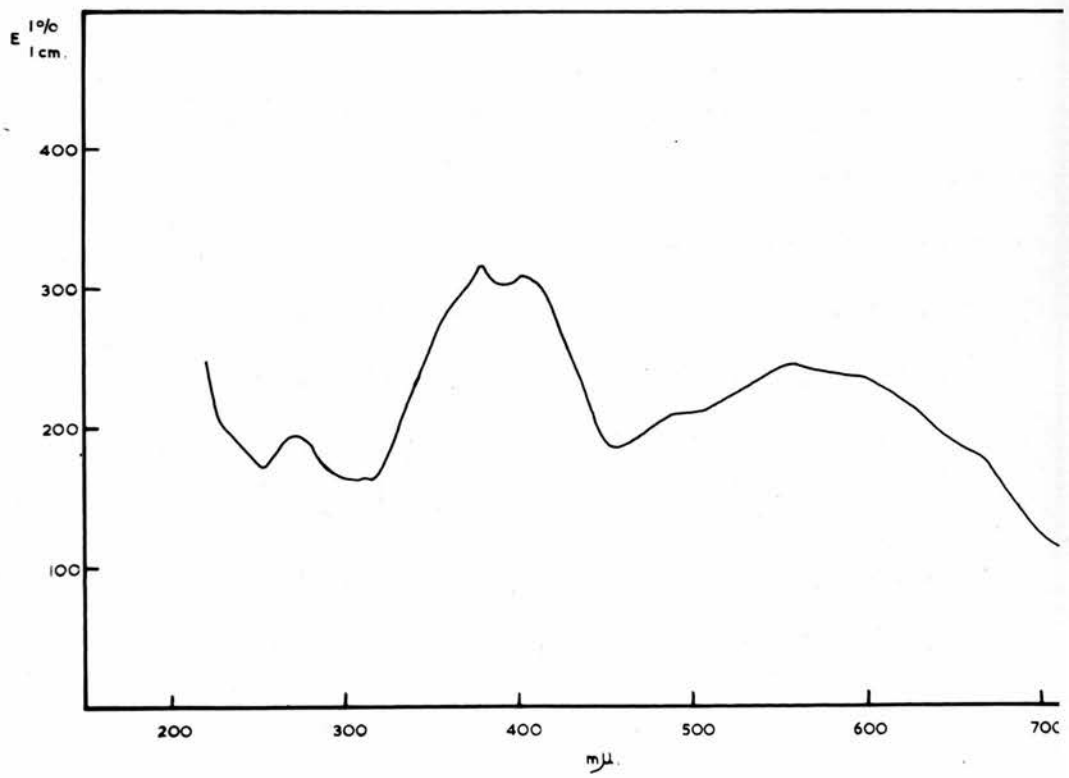


FIG.27 ABSORPTION SPECTRUM OF K_2 IN CONC. H_2SO_4

product after one crystallization from methanol was 145-152°C., identical with that of K_1. On admixture with the latter, there was no depression (145-151°C.).

The absorption spectra in sulphuric acid of equal amounts of dehydroepiandrosterone and its 3β-chloro derivative can be seen in Fig. 26. The identity of the latter was confirmed by analysis:

	Calc.%	Found %
Carbon	74.36	73.79
Hydrogen	8.86	8.31
Chlorine	11.55	12.50

Therefore, K_1 was identified as 3β-chloro-Δ^5-androsten-17-one.

Properties of K_2

This ketone melting at 177-181°C. was obtained from both extracts of boiled urine. Its chromatographic properties were very similar to those of Xa, which was actually present in the first crystals obtained, as shown by paper chromatography. The purified substance, however, did not reduce blue tetrazolium. No formaldehyde was liberated by treatment of K_2 with periodic acid. An aliquot of 99 μg. of K_2 gave a negative

Zimmermann/

Zimmermann reaction with an O.D. = 0.000 in the range 400-550 mμ. These negative results permit the exclusion of the groupings CH_2OH-CO-, CH_2OH-CHOH-, and -CO-CH_2- [x]. Therefore, K_2 is not an α-ketolic, α-glycolic, or 17-ketosteroid. Very probably it is not a 3-keto- or 20-keto-steroid either, as some colour would have developed in the Zimmermann reaction in such a case.

With concentrated sulphuric acid, K_2 produced an intense purple colour with a green fluorescence. The absorption spectrum of this colour is represented in Fig. 27. The relevant numerical data were as follows:

Maxima /

x Androstan-3β-ol-16-one, however, contains the last grouping in ring D and only gives a very weak Zimmermann test (Heard and Sobel, 1946). Oestrone-16(1,3,5(10)-oestratrien-3-ol-16-one also gives a virtually negative test (Huffmann and Lott, 1954).

Maxima: 271 (196); 378 (315); 400 (309); 560 (245).

Minima: 250 (175); 315 (163); 390 (304); 458 (186).

Inflections: 496 (210); 585?(240); 655? (375).

The first figure is the wavelength; the figure between brackets is the corresponding extinction coefficient ($E_{1\ cm.}^{1\%}$). On the assumption that K_2 is a steroid, the peak at 271 mμ. may be assigned to a hydroxyl group (Bernstein and Lenhard, 1954). The doublet at 378 and 400 mμ. occurs in the spectra of many Δ^{5}-3β-stenols (see Bernstein and Lenhard, 1953). There are not many steroids reacting with sulphuric acid to give a purple colour (max. at 560 mμ.). A recent example is 6β-hydroxycortisol diacetate which in sulphuric acid develops a transient violet-purple colour, but no green fluorescence (Burstein and Dorfman, 1955).

The ultraviolet absorption spectrum of K_2 in ethanol revealed a maximum at 242 mμ. The molecular extinction coefficient (assuming a molecular weight of 340) was only 680. Compounds with an α,β-unsaturated ketonic group have a molecular extinction coefficient in the order of 16,000 at that wavelength. Therefore, it seems clear that K_2 does not have this grouping, the absorption/

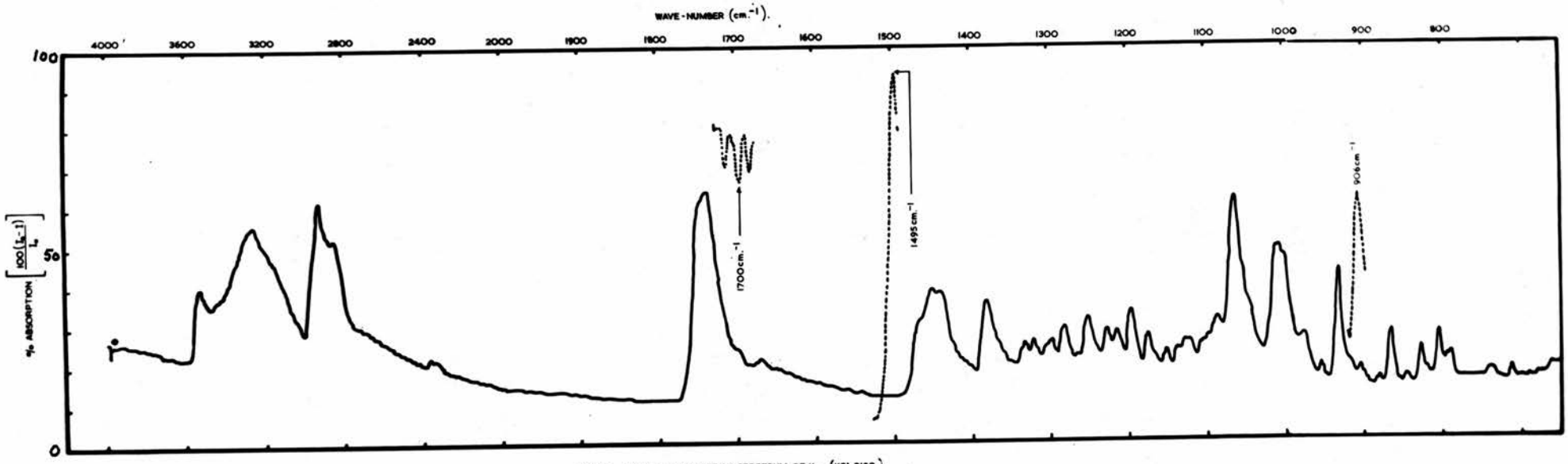


FIG. 28. INFRARED ABSORPTION SPECTRUM OF K_2 (KCl DISC.).

absorption being due to some impurity present in about 4% concentration. There was a faint indication of absorption between 290-300 mμ. (molecular extinction coefficient = 51) which might be due to the same impurity. No absorption at all was observed beyond 320 mμ.

An analysis of 1.898 mg. of K_2 gave:

	%
Carbon	74.23
Hydrogen	8.78
Oxygen	16.99 (by difference).

A reasonable empirical formula might be $C_{19}H_{26}O_3$ (C = 75.46%; H = 8.67%; O = 15.87%) but the agreement is not really good. Another possibility is $C_{23}H_{32}O_4$. This might be the monoacetate of a compound with the formula $C_{21}H_{30}O_3$, but the evidence referred to later does not support this hypothesis.

An infrared absorption spectrum of 1 mg. of K_2 in a KCl disc was obtained in the National Institute for Medical Research, London, through the kindness of Dr R.K.Callow. A copy of the original record is presented in Fig. 28, and an analysis is offered in Table 12 (p.143). The reviews by Rosenkrantz (1955) and Jones and Dobriner (1949) contain very valuable information, of/

Table 12. Infrared absorption spectrum of K_2 (see also Fig. 28).

WN = Wave-number in cm^{-1}.

A = Per cent. absorption = $100(I_o-I)/I_o$. The letters between brackets (s = strong; m = medium; w = weak) are conventionally used to qualify the height of the peaks (Rosenkrantz, 1955). The reference band is that at 1450 $cm.^{-1}$ approximately, which is present in all steroid spectra, and is due to the methylene groups. A strong peak (s) has the same or more intensity than this band, a medium one (m) more than half its absorption, and a weak band (w) less than half its absorption.

WN	A	WN	A
3540	40.0(s)	1150	22.5(m)
3260	55.0(s)	1125	25.0(m)
2930	61.5(s)	1085	31.0(m)
2850	52.0(s)	1062	61.0(s)
2370	22.0(m)	1010	48.5(s)
1740	63.5(s)	975	26.5(m)
1670	21.0(m)	953	19.0(w)
1450 (doublet)	38.0(s)	932	42.5(s)
1380	35.5(m)	902	18.0(w)
1337	25.0(m)	880	15.5(w)
1320	26.0(m)	867	27.5(m)
1295	25.0(m)	845	16.0(w)
1280	29.0(m)	829	23.0(m)
1252	31.0(m)	806	27.2(m)
1227	28.0(m)	788	21.5(m)
1212	28.0(m)	740	17.5(w)
1195	33.0(m)	712	18.0(w)
1175	26.5(m)		

of which use has been made in the interpretation that follows.

The bands at 3260 and 3540 correspond to the free hydroxyl group. The bands at 2930 and 2850 are due to the stretching vibrations of the C-H bond. The band at 1740 suggests a cyclopentanone; as K_2 is not a 17-ketosteroid (negative Zimmermann reaction) a reasonable suggestion for the position of the carbonyl group is C-16. The doublet at 1450 is due to methylene groups and is present in all steroid spectra. The appearance as a doublet is due to the bending vibrations of the methyl group which causes absorption at a slightly lower frequency. In the 'fingerprint' region (1200-700), K_2 shows strong absorption at 1062, 1010, and 932, besides many other peaks of medium or weak intensity.

It is not easy to account for the peaks of medium intensity outside the 'fingerprint' region. The band at 1670 could be assigned to a 5,6-double bond (Rosenkrantz, 1955), but other structures might be responsible for it as well. Many inferences of a negative character can be made from the absence of certain peaks regarded as specific for certain structures. Thus,

all/

all 3β-chloro- Δ^5-unsaturated steroids examined by Hirschmann (1953) showed a strong peak near 761. The absence of such a peak in the spectrum of K_2 permits its exclusion from this group of compounds. Many other groups can be similarly excluded: acetylated hydroxyl groups (absence of band at 1250 of comparative intensity to that at 1740), progesterone-like side-chain (absence of band at 1357), 3-keto and 17-keto groups (absence of medium peaks at 1422 and 1408 respectively) etc.

To summarize the evidence from the infra-red spectrum: K_2 is very probably a steroid with at least one free hydroxyl group and a ketonic group in C-16. There is some evidence for its being a Δ^5-unsaturated compound, although this is not conclusive.

Lack of material prevented further tests for complete identification.

Discussion

The isolation of Xa, the acid-treated product of X which is the most important reducing material liberated from urine by boiling, has not been achieved so far. There is some reason to believe/

believe that the lability of this substance is partly responsible for this failure. For instance, after K_2 had been crystallized from the chromatographic fractions which, it might be expected, would contain Xa, no reducing material was detected in the mother liquors. From the visual inspection of the spots on papergrams obtained at different stages in the isolation procedure, one is led to conclude that the decrease in Xa content is greater than could be reasonably accounted for by the usual losses during the handling of the extract.

Any assessment of the physiological significance of this reducing material must necessarily await its final isolation and the elucidation of its structure; work in this direction is still being continued. Nevertheless, some comments and speculation may be of interest.

In the first place, it should be emphasized again that the chromatographic and other properties of X do not correspond to any of the α-ketolic steroids so far isolated from urine. It will also be remembered that ACTH appears to stimulate the excretion of X, thus providing rather strong evidence to support the hypothesis that/

that X is a corticosteroid. Whether X is in any way related to the eosinopenic material present in boiled urines of ACTH-treated patients (Speirs et al., 1951) cannot be ascertained at the present moment, for no biological testing has yet been conducted on our extracts.

In the preceding section the data concerning some chemical properties of Xa have been described. There is strong but not complete evidence for Xa being a Δ^5-3β-stenol. The isolation from this boiled urine of 3β-chloro-Δ^5-androsten-17-one - a well-known artifact of dehydroepiandrosterone (see Butenandt and Dannenbaum, 1934) - can be accounted for by the treatment with hydrochloric acid in the hydrolysis of the Girard hydrazones. The other ketone isolated, K_2, may very probably be another Δ^5-3β-stenol, judging from some of the maxima shown by the ultraviolet absorption spectrum in sulphuric acid and the infrared absorption spectrum.

If Xa is, as it appears likely to be, a compound with the same structure in rings A and B as dehydroepiandrosterone, its isolation and identification becomes a matter of great interest. Apart/

Apart from dehydroepiandrosterone (Butenandt,1931), eight other Δ^5-3β-stenols have been isolated from urine, and pregnenolone from swine testes (Ruzicka and Prelog, 1943). Six of the urinary compounds: Δ^5-androstene-3β,17β-diol, Δ^5-androstene-3β,16α,17β-triol, Δ^5-pregnene-3β,20α-diol, Δ^5-pregnene-3β17α-diol-20-one, Δ^5-pregnene-3β,16α,20α-triol, and Δ^5-pregnene-3β,17α,20α-triol, were isolated by Hirschmann and Hirschmann (1950) from the urine of a boy with adrenocortical carcinoma. Recently, Fukushima et al.(1954) have found Δ^5-androsten-3β-ol-7,17-dione in normal and abnormal urines. It will be noticed that none of these compounds would reduce blue tetrazolium. The only urinary Δ^5-3β-stenol with this property is 21-hydroxy-pregnenolone, a steroid excreted by normal subjects after ACTH administration, and by patients with adrenal tumours (Dobriner et al., 1950). This compound is said to be present in adrenal gland extracts, but no experimental details are given (Dobriner and Lieberman, 1952). It will be recalled that direct comparison of the running rates of this substance with our reducing material has shown them to be different.

The significance of the presence of these compounds/

compounds in urine is by no means clear. The commonly accepted opinion (Lieberman and Teich, 1953) is that they are metabolites of an intermediate - probably pregnenolone - in the biosynthesis of the adrenal hormones. This opinion is substantiated by the knowledge recently acquired about the role of pregnenolone as a precursor of corticosteroids in the perfused adrenal gland. The enzymic conversion of Δ^5-3β-stenols into Δ^4-3-ketosteroids may take place in several endocrine tissues: placenta, corpus luteum, testis, and adrenal gland (Samuels et al., 1951). In adrenal tumours there might be some difficulty for this conversion to take place, the result being that the generally accepted precursor, pregnenolone, would accumulate and serve as substrate for other unaltered oxidizing systems such as those responsible for the introduction of hydroxyl groups at C-17 or C-21. In view of the large amounts of Δ^5-3β-stenols excreted by the patient studied by the Hirschmann's, it is however difficult to accept this failure in the conversion into α,β-unsaturated ketosteroids as the sole explanation. It is more likely that the production of Δ^5-3β-stenols by the adrenal tumour is considerably increased as well/

well.

An alternative explanation to account for the presence of this type of steroid in urine would be to regard them as metabolic products of the adrenal hormones. However, the reduction of Δ^4-3-ketosteroids to Δ^5-3β-stenols has not been demonstrated so far in any living system, and therefore the first hypothesis is more plausible.

As for the unidentified ketone K_2, which might be considered to be a 16-ketosteroid on the evidence of its infrared spectrum, very little else can be added. Heard and McKay (1939) have isolated androstan-3β-ol-16-one from pregnant mare urine. If on further work the identity of K_2 as a 16-ketosteroid could be confirmed, it would be the first neutral steroid of its kind to be isolated from human urine. Nothing is virtually known about the significance of this kind of steroid.

Summary

a) The ketonic fractions of extracts from boiled male urine have been studied by means of adsorption chromatography.

b) The isolation and identification of 3β-Cl-Δ^5-androsten/

androsten-17-one from one of the ketonic fractions is described.

c) The isolation and some properties of another unidentified ketone are reported. This ketone (K_2) has a melting point (uncorr.) of 177-181°C. after three crystallizations from methanol; it does not reduce blue tetrazolium; no formaldehyde is liberated after oxidation with periodic acid; the Zimmermann reaction is negative; with concentrated sulphuric acid an intense purple colour with green fluorescence is produced. The absorption spectra in the ultraviolet range indicates that it is not a Δ^4-3-ketosteroid. On the basis of the carbon and hydrogen analysis it is assumed that K_2 belongs to the group of $C_{19}O_3$ steroids. The evidence from the intrared absorption spectrum is that K_2 contains a hydroxyl group and a ketonic group, the latter possibly at C-16.

d) The significance of the urinary Δ^5-3β-stenols, found by other authors in large amounts in cases of adrenal tumour, is discussed in connection with the findings reported here and with the probable identity of Xa.

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